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Loss of PTEN-induced kinase 1 (*Pink1*) reduces hippocampal tyrosine hydroxylase and impairs learning and memory

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Abstract

Phosphatase and tensin homolog (PTEN)-induced kinase 1 (Pink1) is involved in mitochondrial quality control, which is essential for maintaining energy production and minimizing oxidative damage from dysfunctional/depolarized mitochondria. Pink1 mutations are the second most common cause of autosomal recessive Parkinson's disease (PD). In addition to characteristic motor impairments, PD patients also commonly exhibit cognitive impairments. As the hippocampus plays a prominent role in cognition, we tested if loss of Pink1 in mice influences learning and memory. While wild-type mice were able to perform a contextual discrimination task, age-matched *Pink1* knockout (*Pink1*^{-/-}) mice showed an impaired ability to differentiate between two similar contexts. Similarly, *Pink1*^{-/-} mice performed poorly in a delayed alternation task as compared to age-matched controls. Poor performance in these cognitive tasks was not the result of overt hippocampal pathology. However, a significant reduction in hippocampal tyrosine hydroxylase (TH) protein levels was detected in the *Pink1*^{-/-} mice. This decrease in hippocampal TH levels was also associated with reduced DOPA decarboxylase and dopamine D2 receptor levels, but not post-synaptic dopamine D1 receptor levels. These presynaptic changes appeared to be selective for dopaminergic fibers as hippocampal dopamine beta hydroxylase, choline acetyltransferase, and tryptophan hydroxylase levels were unchanged in *Pink1*^{-/-} mice. Administration of the dopamine D1 receptor agonist SKF38393 to *Pink1*^{-/-} mice was found to improve performance in the context discrimination task. Taken together, our results show that Pink1 loss may alter dopamine signaling in the hippocampus, which could be a contributing mechanism for the observed learning and memory impairments.

Keywords

dopamine; D1 receptors; hippocampus; Pink1; tyrosine hydroxylase

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Competing Interests

The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Introduction

Brain function, neuroplasticity, and neuronal survival are intimately linked to a continuous supply of energy. Mitochondria use glucose and oxygen extracted from the blood to make ATP, which is used as the energy source to carry out various cellular functions in the brain. During the process of ATP synthesis, reactive oxygen species are also generated that can damage mitochondria, resulting in additional ROS production, compromised ATP synthesis and cellular damage. In order to minimize cellular injury, damaged mitochondria (or portions of a damaged mitochondrion) are removed and degraded by a process referred to as mitophagy.

Pink1 is proposed to play a key role in regulating mitochondrial quality control by identifying damaged mitochondria and targeting them for mitophagy. To accomplish this, it has been hypothesized that Pink1 may serve as a pro-fission signal (Pryde et al., 2016). In healthy, functional mitochondria, Pink1 is constitutively imported into mitochondria where it is cleaved, released back into the cytosol, and degraded. However, if the membrane potential of the inner membrane is compromised (e.g. when damaged), the mitochondrion undergoes fission to isolate the damaged components for subsequent degradation. In these depolarized mitochondria, Pink1 is stabilized and accumulates on the mitochondrial outer membrane. Pink1 then phosphorylates Parkin, an E3 ubiquitin ligase, as well as ubiquitin on the outer mitochondrial membrane. Phosphorylation of Parkin activates its ubiquitin ligase activity, leading to the ubiquitination of mitochondrial proteins, tagging the mitochondrion for degradation (Kane et al., 2014; Koyano et al., 2014).

Loss-of-function mutations in *PARK6*, the gene that encodes the protein PINK1, are responsible for the autosomal recessive variant of Parkinson's disease (PD). Cognitive impairments are among the most frequently reported non-motor symptoms in PD, with dysfunction in executive skills (e.g. attention, planning, and working memory), visuospatial skills (e.g. facial recognition, figure drawing), and memory (e.g. episodic and implicit memory) being affected (Kehagia et al., 2010; Papagno and Trojano, 2018). These cognitive impairments can be observed prior to the manifestation of clinically diagnosed PD based on motor dysfunction (Getz and Levin, 2017). However, while the majority of research using *Pink1* knockout (*Pink1*^{-/-}) animals has focused on motor impairments, the consequences of loss of *Pink1* on cognitive dysfunction has largely not been addressed. As the hippocampus is a key structure for learning and memory, we examined if loss of *Pink1* alters hippocampal physiology and hippocampus-dependent learning and memory. We present results to indicate that the loss of *Pink1* reduces immunoreactivity in the hippocampus of the rate-limiting enzyme for dopamine biosynthesis, tyrosine hydroxylase (TH), as well as DOPA decarboxylase and the dopamine D2 receptor. *Pink1*^{-/-} mice performed poorly in hippocampal-dependent cognitive tasks, a deficit that was rescued by administration of the dopamine D1 receptor agonist SKF38393.

Materials and Methods

Materials

Antibodies used for immunohistochemistry were as follows: vGlut1 (#135303, Synaptic Systems, Gottingen, Germany), vGlut2 (#135403, Synaptic Systems, Gottingen, Germany), bassoon (#141003, Synaptic Systems, Gottingen, Germany), tyrosine hydroxylase (#ab112, AbCam, Cambridge, MA) and NeuN (#MAB377; Millipore, Temecula, CA). For westerns, DOPA decarboxylase (#369003, Synaptic Systems, Gottingen, Germany), dopamine β -hydroxylase (#ab209487, AbCam, Cambridge, MA), tryptophan hydroxylase 2 (#ab184505, AbCam, Cambridge, MA), dopamine D1 receptor (#ab216644, AbCam, Cambridge, MA), dopamine D2 receptor (#ab85367, AbCam, Cambridge, MA), dopamine transporter (#284003, Synaptic Systems, Gottingen, Germany), Lys63-linked ubiquitin (#05-1313, Millipore, Temecula, CA) and beta-actin (#a2228, Sigma, St. Louis, MO) antibodies were used.

Animals

All animal protocols were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC). Homozygous male *Pink1*^{-/-} (B6.129S4-*Pink1*^{tm1Shn/J}) mice in a C57BL/6 background were originally generated from parents with a targeted germ-line deletion of exons 4–7 and a nonsense mutation at exon 8 (Kitada et al., 2007) and knockout animals were purchased from Jackson Laboratories (JAX stock #017946; Bar Harbor, ME). This homozygous knockout mouse strain is maintained by Jackson Laboratories using a homozygote $\times$ homozygote breeding scheme, and has been backcrossed to the C57BL/6J line for at least 8 generations. As a consequence of their breeding scheme, wild-type siblings are not commercially available from Jackson Laboratories. Age-matched C57BL/6J mice are recommended for use as control animals. All mice were individually housed upon receipt (6–10 weeks of age) to avoid barbering. Animals used for motor and behavioral analyses were tested in all tasks. All studies were performed by blinded observers, and animals were only identified by a numeric code.

Isolation of mitochondria

To isolate mitochondria from brain tissues, Percoll density gradient centrifugation was used as previously described (Lennartz, 2008). Hippocampi from two animals per experimental group were pooled together to increase the quantity of starting material. Tissue was homogenized in ice-cold isolation buffer (100 mM Tris pH 7.4, 10 mM EDTA, 12% Percoll solution, 1 mM sodium fluoride, 1 mM sodium molybdate, 100 nM okadaic acid, 1 mM PMSF and 10 μ g/ml leupeptin) using 4 strokes in a Dounce homogenizer using the loose pestle followed by 8 strokes using the tight pestle. A small fraction of each homogenate was removed for determination of protein content. The remaining homogenate was then layered onto a discontinuous Percoll gradient (26% and 40% Percoll) and centrifuged for 10 minutes (30,700 $\times$ g at 4°C). The enriched mitochondrial fraction was removed from the 26%:40% Percoll interface, transferred to clean centrifuge tubes, and diluted (1:4) with isolation buffer. Fractions were then pelletized by centrifugation (16,700 $\times$ g at 4°C) for 10 minutes. The supernatant was discarded and the samples were either immediately prepared for electron

microscopy analysis or snap frozen and stored at -80°C . Samples underwent one freeze-thaw cycle prior to western analysis.

Transmission electron microscopy

Immediately after isolation, mitochondria from mice hippocampi were applied to freshly glow-discharged (30 sec) carbon-coated copper grids, blotted, and then fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 15 minutes on a chilled plate. Grids were prepared for negative staining by washing once in HBS (HEPES buffered saline), then three times with water, followed by incubation in methylamine vanadate (Nanovan, Nanoprobes) for 20 seconds. Grids were then blotted and dried for at least 10 minutes prior to imaging. CCD images of isolated mitochondria were taken on a JEOL1400 transmission electron microscope running at 120 kV with a Gatan Orius SC1000 camera. Four to six grid squares were selected based on optimal density of structures within the grid square and high magnification images (15kx) of the entire grid square were taken to capture all mitochondria within the selected grid square.

Mitochondrial length measurements

To remove potential biases, the experimenters quantifying the length of individual mitochondria were blinded to the sample identities. To quantify mitochondrial length, CCD images were displayed in FIJI (NIH) and a line was drawn along the major axis of each mitochondrion. Length measures and aspect ratios (major/minor axis) were used for further analysis. Measures were extracted for 200 mitochondria/animal, separated by length into 500 nm bins, and summed across the six animals per group.

Immunohistochemistry

Mice were euthanized by sodium pentobarbital overdose (100 mg/kg) then transcardially perfused with ice-cold saline followed by 4% paraformaldehyde in PBS. Brains were removed and post-fixed in perfusant for 24 hours before step-wise cryopreservation in sucrose (15% to 30% in PBS). Cryo-sections (40 μm thick) containing the dorsal hippocampus were permeabilized in 0.25% Triton-X100/PBS and blocked in 2.5% normal goat serum/2.5% BSA/PBS before incubation in primary antibody solutions overnight at 4°C . Tissues were then washed in PBS, incubated in species-specific secondary antibody solutions, washed again in PBS, and allowed to dry overnight before coverslipping with Fluoromount G (Southern Biotech, Birmingham, AL). Immunoreactivity within the hippocampus was assessed by carefully outlining the hippocampus and determining the mean fluorescent intensity value using *Image J* after subtraction of background. Three sections/animal, corresponding to approximately -2.0 ± 0.2 mm from bregma, were quantified and averaged.

Cell Counts

Calbindin-D28k-positive cells in the dentate gyrus were counted using *Stereo Investigator* (MicroBrightField, Williston, VT). The dentate gyrus was carefully outlined by an experimenter blind to the group designations. Cap zones of 2.5 μm were used to avoid counting cell caps. The counting grid was 100×100 μm using a counting frame of 25×25

μm. These parameters were chosen based on preliminary counts in order to “mark” 100–150 cells/section. Calbindin-D28k-positive cells in 3 sections/animal, corresponding to approximately -2.0 ± 0.2 mm from bregma, were counted and averaged.

Western Analysis

Mice were deeply anesthetized with isoflurane, exsanguinated, and hippocampal tissues dissected under ice cold artificial cerebral spinal fluid (aCSF) and snap frozen on dry ice. Total protein homogenates were prepared using a Potter-Elvehjem homogenizer in lysis buffer containing 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4 , 5 mM NaF, 5 mM Na_2MoO_4 , 1 mM DTT, and 1X cOmplete protease inhibitor cocktail (Sigma-Aldrich; St. Louis, MO). Total protein concentration was measured by bicinchoninic acid (BCA) assay using BSA as the reference standard. Sample aliquots were diluted into 1X *Wes* sample buffer and protein content equalized. Target proteins were quantified using an automated capillary immunoassay system (*Wes* system; Protein Simple, San Jose, CA). Immunoreactivity was detected with a luminol-peroxide solution and a series of timed exposures were collected. The images were analyzed using *Compass* for SW (version 3.1.7; Protein Simple). Results were normalized against Actb, and presented as percent control.

Immunoprecipitation

Tissues were homogenized as described in the Western Analysis section, and 100 μg extract was diluted into 1 ml of RIPA buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl) containing protease and phosphatase inhibitor cocktails (Thermo Scientific, Waltham, MA). Diluted extracts were precleared for 3 hr by incubation with 20 μl protein A-agarose (Thermo Scientific, Waltham, MA), followed by overnight incubation (on a rotator) in 20 μl protein A-agarose and 5 μg phospho-TH (ser40) antibody at 4°C. Agarose beads were collected by centrifugation (5 min, 1,000 x g) and washed five times with RIPA buffer. Following the final wash and bead collection, the bound proteins were removed by incubation in 1X *Wes* sample buffer for 10 min at 65°C. These extracts were then used for western analysis to examine K63-linked ubiquitination and TH levels.

RNA isolation and quantitative PCR

Tissues from the hippocampus, striatum, and region containing the ventral tegmental area/substantia nigra were quickly dissected under ice-cold aCSF and snap frozen on dry ice. Tissues were homogenized on ice in a Potter-Elvehjem homogenizer in lysis buffer as described in the Western Analysis section, and immediately after disruption a portion of the homogenate was diluted 1:5 into a solution containing 4 M guanadinium thiocyanate, 25 mM $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$, 0.5% sarcosyl, 100 mM 2-mercaptoethanol. Total RNA was then isolated by phenol:chloroform extraction and ethanol precipitation (Chomczynski and Sacchi, 1987). RNA was quantified by spectrophotometry, and cDNA was generated from either 1 μg (hippocampus) or 0.5 μg (striatum, VTA/SN) total RNA using the High Capacity cDNA Reverse Transcription kit in a 20 μl reaction following the manufacturer's protocol. Tyrosine hydroxylase mRNA levels were quantified by Taqman probe (Applied Biosystems) using a CFX Connect (Biorad Instruments). Data was normalized against internal reference RNAs (Actb and Gapdh) for each sample using the formula $C_T = C_T(\text{TH}) - C_T(\text{average})$

Actb:Gapdh), then C_T values were calculated using the wild-type striatum as the reference. Data is presented as the group mean $\pm$ SEM.

Wire hang task

Grip strength was tested using the wire hang task. A 2 mm gauge wire was suspended in place from a height of 35 cm. For each trial, the animal was allowed to hang until falling. Animals were tested in three trials with a 10 minute inter-trial interval.

Rotarod

Rotarod performance was assessed using an ENV-576 Rotarod from Med Associates (Fairfax, VT). Mice were placed by hand on the rotating rod, facing away from the direction of rotation. The initial rotation speed started at 4 rpm and gradually increased to 40 rpm over a 4 minute period. The time the animal remained on the beam without falling off (or if the animal failed to continue walking but held onto the rotating rod for three complete rotations) was recorded. Each mouse was given 3 trials per day, which were averaged.

DigiGait Analysis

Gait analysis was carried using a DigiGait (Framingham, MA). Mice were placed on the transparent treadmill and forced to walk/run at a fixed velocity of 20 cm/sec on a level gradient. Bumpers in front and behind the animal are used to restrict the mouse and discourage “riding” on the treadmill. Bumper separation was 15 cm. A high-speed camera (150 fps) aimed at the ventral surface of the mouse recorded paw steps which were digitized for analysis. Settings used for defining the paw area were adjusted, as needed, to ensure fidelity of the measures. Mice were initially allowed to run on the treadmill for a period of 2 seconds, followed by a 10 second rest period. This procedure was repeated 3 times to ensure that all animals would run on the treadmill. For gait analysis, mice were run on the treadmill for a period of 8–10 seconds, and a 3 second video was selected (based on mouse being centered in the camera field and actively walking) for analysis. Gait metrics (stance width, angle of paw, stride length, braking speed, propulsion speed, and swing speed) for each paw were collected separately. Forepaw and hind paw measures were averaged within an animal before comparison across groups.

Spontaneous alternation

Spontaneous alternation was tested in a plus maze consisting of four 30 cm $\times$ 6.5 cm $\times$ 10 cm (L $\times$ W $\times$ H) closed arms connected to a central hub. Each animal was tested in a single 8 minute trial in which the mouse was placed in the central hub and allowed to freely explore the maze. Movement in the maze was recorded using a CCD camera. The maze was cleaned with 70% alcohol between animals. An arm entry was counted if the animal’s entire body, including the tail, entered the arm. Reentries, wherein an animal immediately re-enters the same arm it just left, were excluded (Lennartz, 2008). Alternation was calculated using the method of Ragozzino et al. (1996), wherein a successful alternation was counted if in five consecutive arm entries, the animal chose four different arms (Ragozzino et al., 1996). The percent alternation was calculated as the number of successful alternations out of the total number of possibilities.

Context Discrimination

The context discrimination task was adapted from the contextual fear conditioning protocols previously described by Frankland, 1998 (Frankland et al., 1998). Prior to initiating training, independent wild-type and *Pink1*^{-/-} mice were assessed for their shock threshold, essentially as described previously (Rodríguez and Wetsel, 2006). Both groups displayed overt reactions to the shock at the 0.3 mA threshold. A 0.7 mA shock (>2X threshold) was employed to generate a fear response. The context discrimination task consists of two conditioning chambers (a “shock” and a “safe” chamber), which share some features (e.g. size, shape) but differ in others (e.g. visual cues, smell, and flooring). Animals were first pre-exposed to each chamber one day prior to training. Training consisted of morning and afternoon sessions, during which the animals were exposed to either the shock chamber or safe chamber in alternating sessions. A mixed exposure protocol was used, in which half of the animals were exposed to the shock chamber in the morning session then exposed to the safe chamber in the afternoon session, while the other half were exposed to the safe chamber followed by the shock chamber. In the shock chamber, animals received a mild foot shock (2 s, 0.7 mA) 148 sec after being placed in the chamber. Animals remained in the chamber for an additional 30 sec before being returned to their home cage. In the safe chamber, animals again remained in the chamber for a total of three minutes, but no foot shock was delivered. This training procedure was repeated for four days. An overhead CCD camera creates an image every 100 msec which is segmented into individual pixels for analysis of freezing behavior using *Ethovision XT* software (Leesburg, VA, USA). Movement was defined as a change in 0.1% of the pixels. To ensure the accuracy of these measures, random videos were hand-scored by an investigator. The percent of time the animals remained frozen within each chamber was used as an index of fear memory. The difference in freezing response between the shock and safe cages was used as an indicator of discrimination.

Marble burying and open field tasks

Anxiety was measured using the marble burying and open field tasks. The marble burying test as described in Angoa-Perez et al. (2013) was used to measure anxiety-like behaviors (Angoa-Perez et al., 2013). Standard polycarbonate rat cage with fitted filter-top covers were filled with unscented mouse bedding material to the depth of 5cm. Twenty standard black glass marbles (15 mm diameter, 5.2 g) were placed gently on the surface of the bedding in 5 rows of 4 marbles. The mouse was then allowed to remain undisturbed in the cage for 30 minutes. After test completion, two scorers (blind to the genotype of the mouse being tested) counted the number of marbles buried. A marble was considered buried if two-thirds of its surface area was covered by bedding. Fresh bedding was used for each animal. Open field testing was conducted in an 18 × 18 inch container. Mice were allowed to explore the chamber for a period of 5 minutes. Movement in the maze was tracked using *Ethovision XT* software. For analysis, the chamber was segmented into 36 equal sized bins. The number of entries and the time spent in the center of the open field (12 × 12 inch; comprised of 16 bins) was compared to assess anxiety-like behavior.

Preparation and injection of SKF38393

SKF38393 stock (20 mg/ml) was prepared in saline and diluted prior to injection to give a final solution containing 0.25 mg/ml SKF38393. For testing performance in spontaneous alternation, mice received either 1.0 mg/kg SKF38393 or an equal volume of vehicle (i.p.) 30 minutes prior to testing. For testing in the contextual discrimination task, each mouse received 1 mg/kg SKF38393 or saline 30 min prior to each training session (i.e. prior to being placed in either the “shock” or “safe” cage). Thus, for the contextual discrimination task, each mouse received 2 injections/day, for a total of 8 injections over the four training days.

Statistical Analysis

All data was subjected to a Shapiro-Wilk normality test and an equal variance test prior to analysis. Cell counts, fluorescent intensity and western data were analyzed across groups using a two-tailed Student's t-tests for unpaired variables. Mitochondria data was binned into groups based on length, and compared across groups using a Chi Square test. For assessing spontaneous alternation across conditions (pre, SKF38393 treatment, and washout) between wild-type and *Pink1*^{-/-} mice, a two-way repeated measures ANOVA was used, followed by *post-hoc* analysis with correction for multiple comparisons. For context discrimination, freezing behavior recorded within a group was analyzed across time and between contexts using a repeated measures two-way ANOVA. Results were considered significant at $p < 0.05$. Data are presented as the mean $\pm$ SEM.

Results

Loss of Pink1 does not significantly alter mitochondrial length in the hippocampus

It has been previously reported that mitochondria in the striatum are morphologically normal in either adult (3–4 month) or aged (22–24 month) *Pink1*^{-/-} mice, with an increase in the number of longer mitochondria (Gautier et al., 2008). As the hippocampus plays a critical role in learning and memory, we therefore questioned if loss of Pink1 alters mitochondrial size in the hippocampus. Mitochondria were isolated from the hippocampi of 9–11 month old WT (n=6) and *Pink1*^{-/-} (n=6) mice and analyzed by transmission electron microscopy (TEM) imaging. Representative images of isolated mitochondria are shown in Fig. 1A. Measurement of mitochondria length (1200 mitochondria; 200 mitochondria/animal) was carried out by an experimenter unaware of the group designations. Fig. 1B shows that when the mitochondria were grouped into 0.5 μ m length bins, no significant difference in the number of mitochondria was observed between the two groups ($X^2=11.398$, $p = 0.077$). These data indicate mitochondrial length in the hippocampus was not affected by *Pink1*^{-/-}.

Pink1^{-/-} mice do not have gross hippocampal pathologies or neuronal loss

We next examined if the loss of Pink1 caused overt loss of hippocampal neurons. Fig. 2A shows representative photomicrographs of hippocampi from age-matched WT and *Pink1*^{-/-} mice immunostained for the neuronal marker NeuN. Evaluation of NeuN immunoreactivity did not reveal any visible cell loss at either low or high magnification.

It has been previously reported that loss of Pink1 reduces the number of doublecortin-positive newborn neurons in the hippocampus (Agnihotri et al., 2017). Consistent with this observation, we also observed a significant reduction in the number of doublecortin-positive newborn neurons (Supplemental Fig. 1), which could reduce the number of mature granule neurons. To examine this, mature granule neurons were identified by their expression of the calcium binding protein calbindin-D28k (Fig. 2B). Using unbiased stereology, we counted the number of calbindin-D28k-positive neurons in age-matched WT (n=5) and *Pink1*^{-/-} (n=5) mice. Loss of Pink1 was not associated with a decrease in the number of calbindin-D28k-positive neurons (t=1.742, p=0.125) compared to WT controls.

As the expression of the synaptic vesicle proteins vGlut1, vGlut2, and Bassoon have been found to be altered in models of Parkinson's Disease, and changes in the levels of these proteins have been implicated in cognitive decline (Massie et al., 2010; Yabe et al., 2018), we also examined the immunoreactivity of these proteins in the hippocampi of wild-type and *Pink1*^{-/-} mice (n=4/group). No overt immunostaining differences were observed between the two groups (Fig. 2C). Quantification of vGlut1 (t=-0.144, p=0.891), vGlut2 (t=-0.456, p=0.668), and Bassoon (t=-0.0246, p=0.979) immunoreactivities did not reveal any differences between the groups.

Loss of Pink1 does not cause motor dysfunction

As PD is characterized by motor impairments, mice were tested for their muscle strength and motor coordination using the wire grip and rotarod tasks, respectively. All *Pink1*^{-/-} (n=10) and wild-type (n=10) mice used in these studies were 9–11 months of age. Although the *Pink1*^{-/-} mice were smaller than their wild-type counterparts (Supplemental Fig. 2), Fig. 3A shows that when tested in the wire grip task, both wild-type and *Pink1*^{-/-} mice were capable of hanging from the wire for comparable amounts of time ($T = 122.00$; $p = 0.212$). Similarly, there was no significant difference in the ability of the *Pink1*^{-/-} mice to perform the rotarod task compared to age-matched wild-type controls ($F = 0.821$, $p = 0.377$), with both groups showing improved performance across days ($I = 15.492$, $p < 0.001$; Fig. 3B).

To examine more subtle differences in motor function, WT and *Pink1*^{-/-} mice were tested on a DigiGait gait analyzer (Mouse Specifics, Inc., Framingham, MA). The DigiGait uses a high-speed camera to record and digitize each paw as the mouse runs on a clear treadmill (Fig. 3C). From these images, the position of each paw is mapped over time to allow calculation of metrics such as stance width and paw angle (Fig. 3C), as well as stride length, braking time, propelling time, and swing time (Fig. 3D). Values for forepaws and hind paws were averaged within each animal and compared across groups (Fig. 3E). No significant differences were observed between the groups on any of the forepaw measures ($F = 0.715$; $p = 0.409$). A significant interaction of group and gait metric was seen in the hind paws that was due to a significant decrease in hind paw angle in the *Pink1*^{-/-} mice ($F = 4.646$, $p < 0.001$). This decrease may be due to the relatively smaller size of the *Pink1*^{-/-} mice compared to the WT mice. Consistent with this premise, when *Pink1*^{-/-} mice were compared to weight-matched WT mice, no significant difference in hind paw angle was observed (t=0.142, p=0.890).

Loss of Pink1 causes learning and memory impairments

To examine if loss of Pink1 influences hippocampus-dependent cognitive performance in the absence of motor impairments, animals were tested in two hippocampal-dependent tasks: spontaneous alternation and context discrimination (Frankland et al., 1998; Kirkby et al., 1967). In the spontaneous alternation task, animals are allowed to freely explore a 4-arm plus maze surrounded by distinct extramaze cues (Fig. 4A). Mice with intact hippocampal function spontaneously alternate the arms that are visited, resulting in each of the 4 arms being entered out of a possible 5 tries (indicated by green boxes). The red box indicates an unsuccessful alternation. The percentage of successful alternations (out of the number of possible attempts) during the 8 minute trial is used as an index of hippocampal working memory function (Ragozzino et al., 1996). Fig. 4B shows that by comparison to wild-type controls (WT, $n=10$), *Pink1*^{-/-} mice (KO, $n=10$) made significantly fewer alternations ($t=-2.966$, $p=0.008$), suggesting impaired hippocampal function. These differences were not due to differences in exploratory behavior or locomotion, as there were no significant differences in either numbers of entries ($t=0.841$, $p=0.411$; Fig. 4C), distance traveled ($t=-0.139$, $p=0.891$; Fig. 4D) or velocity ($t=-0.135$; $p=0.894$; not shown) between the wild-type and *Pink1*^{-/-} mice.

We next tested groups of *Pink1*^{-/-} and age-matched wild-type mice in the context discrimination task. Animals were first pre-exposed to two similar, but not identical, chambers during which no footshock was delivered (Fig. 4E). Contextual discrimination was then carried out over a four day training period in which animals were tested for their ability to discriminate between the training chamber in which a mild footshock was delivered (“shock” context) versus the chamber where animals received no footshock (“safe” context). Fig. 4F shows that during training, wild-type mice ($n=14$) learn to differentiate between the “shock” and “safe” contexts as indicated by a significant difference in freezing behavior in the two contexts over the course of training (interaction of context and trial: $F=15.015$; $p<0.001$). In contrast, *Pink1*^{-/-} animals ($n=13$) display similar freezing behaviors in both the “safe” and “shock” contexts (group main effect: $F=0.342$; $p=0.569$), indicating impaired contextual discrimination (Fig. 4G). As this difference could have resulted from higher anxiety levels in *Pink1*^{-/-} mice, we compared independent groups of *Pink1*^{-/-} and wild-type mice in two tasks that assess anxiety: the marble burying and open field tasks. In the marble burying task, mice experiencing anxiety dig in the bedding, thereby burying the marbles. The results presented in Fig. 4H show that there was no significant difference observed between wild-type and *Pink1*^{-/-} animals in the number of marbles that were buried ($t=0.987$, $p=0.337$), suggesting similar baseline anxiety levels. Fig. 4I shows representative movement traces for a wild-type and *Pink1*^{-/-} mouse in the open field task. No significant differences in either distance traveled ($T=108.00$, $p=0.850$; Fig. 4J) or entries into the center of the arena ($t=1.105$, $p=0.284$; Fig. 4K) were detected. *Pink1*^{-/-} mice tended to spend more time in the center of the arena (Fig. 4L), although this did not reach statistical significance ($t=1.858$, $p=0.080$).

Loss of Pink1 decreases tyrosine hydroxylase levels in the hippocampus

As the cognitive impairments seen in *Pink1*^{-/-} mice were not associated with overt neuronal loss, we examined if loss of Pink1 altered dopamine biogenesis and signaling. When we

examined TH immunolocalization in the hippocampus, a visible decrease of immunoreactivity was observed in *Pink1*^{-/-} mice (Fig. 5A; signal appears as white in this grayscale image). To further examine this, the levels of TH mRNA and protein in the hippocampal tissue were quantitated in *Pink1*^{-/-} mice (n=4) and compared to age-matched WT controls (n=4). For the Wes analysis of TH protein, total protein extracts from the hippocampus (0.25 mg/ml), striatum (0.10 mg/ml), and substantia nigra/ventral tegmental area (0.05 mg/ml) were used. Different starting amounts of total protein were loaded for the individual brain regions in order to be in the linear range of detection. Thus, direct comparisons of TH protein levels across regions cannot be made from these experiments. For the qPCR measurements, cDNA generated from either 1 µg (hippocampus/striatum) or 0.5 µg (substantia nigra/ventral tegmental area) total RNA was used. Figure 5B and 5C shows that a modest, but significant, decrease in hippocampal (Hip) TH protein levels was observed (t=2.475, p=0.048), that occurred in the absence of significant changes in hippocampal TH mRNA (t=1.395, p=0.212; Fig. 5D). No significant changes in either TH protein (t=0.936, p=0.385) or mRNA (t=-1.483, p=0.172) were detected in the striatum (Str) of *Pink1*^{-/-} mice compared to age-matched WT controls. Similarly, no significant changes in either TH protein (t=1.395, p=0.213) or mRNA (t=1.405, p=0.194) were found between genotypes in tissue samples taken from the ventral tegmental area/substantia nigra (VTA/SN), which harbor the cell bodies of dopaminergic neurons that project to the hippocampus.

It has been reported that TH can be degraded by both proteosomal and lysosomal pathways (Congo Carbajosa, et al., 2015). We therefore questioned if TH ubiquitination is increased in the hippocampi of *Pink1*^{-/-} mice. TH was immunoprecipitated using an antibody which specifically recognizes phosphorylated TH (Ser40), as this phosphorylation site has been previously linked to TH ubiquitination (Kawahata, et al., 2015). When the immunoprecipitated proteins were probed with an antibody against K48-linked ubiquitin, no specific bands were observed (data not shown). However, a significant increase in immunoreactivity (filled triangle) was detected (t=-3.808, p=0.004; Fig. 5E) in the *Pink1*^{-/-} mice when the immunoprecipitated material was probed with an antibody against K63 ubiquitin. Interestingly, putative TH breakdown products (open triangles) were observed in the samples from the *Pink1*^{-/-} mice. Consistent with our previous western blot results using independent hippocampal protein extracts, TH levels (filled triangle) in these immunoprecipitated extracts were found to be significantly reduced (t=2.339, p=0.044; Fig. 5E). Similar to that seen with the K63-linked ubiquitin antibody, the TH antibody also cross-reacted with smaller molecular weight proteins (open triangles), suggestive of TH breakdown products.

Loss of Pink1 decreases the levels of presynaptic proteins present in dopaminergic neurons

Our results that *Pink1*^{-/-} mice have reduced hippocampal TH levels suggests that these mice may have a loss of hippocampal dopaminergic fibers. To examine this possibility, the levels of key proteins present in dopaminergic fibers were evaluated (Fig. 6A). Fig. 6B shows representative westerns for DOPA decarboxylase (Ddc), dopamine reuptake transporter (Slc6a3), and dopamine D2 receptors (Drd2; primarily presynaptic) using hippocampal tissue extracts collected from wild-type and *Pink1*^{-/-} mice (n=4/group). Fig. 6C shows

decreased levels of Ddc ($t=3.546$, $p=0.012$), Slc6a3 ($t=4.377$, $p=0.005$) and Drd2 ($t=4.459$, $p=0.004$), but not postsynaptic dopamine D1 receptors (Drd1; $t=0.616$, $p=0.560$) were observed. In contrast to the observed decreases in dopaminergic fiber proteins, the levels of dopamine beta hydroxylase (Dbh, converts dopamine to norepinephrine; $t=1.081$, $p=0.321$), tryptophan hydroxylase (Tph2, the rate-limiting enzyme for serotonin synthesis; $t=1.541$, $p=0.184$) and choline acetyltransferase (Chat, the enzyme responsible acetylcholine synthesis; $t=-1.799$, $p=0.110$) were not significantly different between WT and *Pink1*^{-/-} mice. No significant change in Actb ($t=0.359$, $p=0.732$) was detected, indicating equality in protein loading.

Administration of a dopamine D1 agonist improves spontaneous alternation by *Pink1* knockout mice

Dopamine signaling, especially via the dopamine D1 receptor, has been shown to be involved in a variety of learning and memory paradigms (Edelmann and Lessmann, 2018; Jay, 2003; Kempadoo et al., 2016). As our western blot show that the levels of TH, the rate-limiting enzyme in dopamine synthesis, is decreased in the hippocampus of *Pink1*^{-/-} mice, we tested if enhancing dopamine signaling could improve cognition in *Pink1*^{-/-} mice. To test this premise, we examined the consequences of systemic administration of a D1 receptor agonist using an off-on-washout paradigm. When the percentage of successful alternations were compared across trials between the *Pink1*^{-/-} and wild-type mice, a significant interaction of genotype and trial was observed ($F=7.549$, $p=0.002$; Fig. 7A). *Post-hoc* analysis revealed that this effect was due to a significant difference in performance between the groups during baseline testing (Pre) and the washout test, but not following SKF38393 administration. Within group comparisons revealed that wild-type mice did not significantly differ in their performance over time, showing no difference between baseline, post-drug administration, or washout ($p=0.273$). In contrast, *Pink1*^{-/-} mice had a significant change in performance that was due to an increase in successful alternations when tested acutely after SKF38393 injection ($p=0.027$).

Administration of a dopamine D1 agonist enhances context discrimination by *Pink1* knockout mice

To test if dopamine D1 receptor agonism can improve context discrimination, *Pink1*^{-/-} mice ($n=10$ /genotype/group) were injected with either 1.0 mg/kg SKF38393 or an equal volume of saline (100 μ l) 30 minutes prior to each training session as described in the Experimental Procedures section. *Pink1*^{-/-} mice injected with saline ($n=10$) were unable to differentiate between the two contexts (group main effect: $F=0.528$, $p=0.486$; Fig. 7B). However, as previously seen in the spontaneous alternation task, SKF38393 administration to *Pink1*^{-/-} mice improved context discrimination, as indicated by significantly more freezing behavior in the shock context than the safe context by the end of testing (interaction of context and trial: $F=3.084$, $p=0.028$; Fig. 7C). In contrast, wild-type animals injected with SKF38393 (interaction of context and trial: $F=3.626$, $p=0.014$, $n=10$; Fig. 7D) performed similarly to wild-type mice injected with vehicle (interaction of context and trial: $F=7.037$, $p<0.001$; $n=10$; Fig. 7E).

Discussion

In the present study, we examined the consequences of loss of Pink1 on hippocampal mitochondria, dopamine biogenesis, motor function, and hippocampus-dependent learning and memory. Our results revealed four key findings: 1) Loss of Pink1 did not significantly alter hippocampal mitochondrial length, nor did it cause an overt loss of hippocampal neurons (by gross visual inspection). 2) The levels of several proteins involved in dopamine biosynthesis in the hippocampus of *Pink1*^{-/-} mice are significantly decreased compared to age-matched wild-type controls, 3) *Pink1*^{-/-} mice perform poorly in hippocampal-dependent tasks in the absence of detectable motor impairments, and 4) the observed learning and memory impairments were corrected by treatment with a dopamine D1 agonist.

Parkinson's disease (PD) is a progressive neurodegenerative disease caused by the loss of dopaminergic neurons (McDonald et al., 2018). Clinical presentation of PD includes motor symptoms, such as slow movement, rigidity, and gait disturbances. In addition to movement disorders, PD patients often present cognitive impairments (Aarsland et al., 2017). The most common form of the disease is idiopathic in which symptomology appears late in life. Familial forms of the disease, however, can have early- or late-onset and are inherited as either an autosomal dominant (mutations in *SNCA*, *LRRK2* genes) or an autosomal recessive (mutations in *PARKIN*, *DJ-1* or *PINK1* genes) disease (Hauser et al., 2017). PTEN-induced kinase 1 (*PINK1*) mutations are the second most common (after *PARKIN*) form of autosomal recessive disease (Bonifati, 2014). The average age of disease onset in persons with *PINK1* mutations is 36±12 years (Ishihara-Paul et al., 2008).

The Pink1 protein is proposed to play a key role in mitochondrial quality control by identifying damaged mitochondria and targeting them for mitophagy. To accomplish this, it has been hypothesized that Pink1 may serve as a pro-fission signal (Lisman and Grace, 2005). Based on this action of Pink1, we anticipated that loss of Pink1 would result in changes in the size distribution of hippocampal mitochondria. Contrary to our expectations, we did not detect any significant differences in the size distribution of mitochondria in the hippocampus of *Pink1*^{-/-} versus age-matched WT controls. This result is in contrast to a previous report that found an increase in the number of larger mitochondria within the striatum in *Pink1*^{-/-} mice (Gautier et al., 2008). While the reason for this discrepancy is not clear, it may be related to the structures examined (striatum versus hippocampus), or the techniques utilized (electron microscopy on tissue versus isolated mitochondria).

As PD has been linked to disruptions in dopamine signaling, and hippocampal function can be modulated by dopamine (Johnson et al., 2007; Lisman and Grace, 2005; Shohamy and Adcock, 2010; Wittmann et al., 2005), we questioned if the loss of Pink1 was associated with changes in hippocampal TH levels. We observed that TH protein levels in the hippocampus were significantly decreased in *Pink1*^{-/-} mice compared to age-matched WT controls. It has been previously reported that post-mortem analysis of Parkinson's disease brains have reduced dopaminergic neurons in the VTA as well as reduced TH immunoreactivity within the hippocampal perforant pathway (Torack and Morris, 1992). Although we did not count the number of dopaminergic cells within the VTA, the reduction in TH immunoreactivity we observed in the hippocampus was found to occur in the absence

of detectable changes in either TH mRNA or TH protein in the VTA. However, we did observe an increase in K63-linked TH ubiquitination, suggestive of enhanced lysosomal degradation. However, it is not known if this enhanced degradation was solely responsible for the decrease in TH protein levels we observed.

It is interesting to speculate that dopaminergic fibers in the hippocampus may be selectively vulnerable to the loss of Pink1. Alternatively, as TH is the rate-limiting enzyme for the production of both dopamine and norepinephrine, and dopamine release in the hippocampus has been suggested to arise in part from noradrenergic neurons (Kempadoo et al., 2016), the reduction in TH levels we observed could have arisen from a decrease in noradrenergic fibers originating from the locus coeruleus. However, we did not observe a decrease in hippocampal dopamine beta hydroxylase (the enzyme responsible for converting dopamine to norepinephrine) levels, suggesting that noradrenergic fibers are not decreased in *Pink1*^{-/-} mice. Further, we did not detect any changes in the hippocampal levels of tryptophan hydroxylase or choline acetyltransferase, suggesting that hippocampal serotonergic and cholinergic fibers are not detectably affected by loss of Pink1. While the reason for the loss of dopaminergic fibers in the hippocampus is not known at present, recent studies have reported that Pink1 can phosphorylate the mitochondrial transport protein Miro, and alter mitochondrial motility (Wang et al., 2011). As mitochondrial transport is essential for neuronal function and survival, the observed loss of TH fibers in *Pink1*^{-/-} mice could have resulted from, at least in part, impaired mitochondrial motility. Future studies will be required to address this possibility.

As TH is the rate-limiting enzyme for dopamine synthesis, decreased TH protein (or loss of TH-positive fibers) in the hippocampus is likely to reduce dopamine levels and downstream signaling. This suggests that strategies to increase dopamine signaling may improve hippocampus-dependent learning and memory in *Pink1*^{-/-} mice. Although D2 receptor antagonists are not typically prescribed in Parkinson's disease (due to a concern of further reducing dopaminergic signaling), D2 receptor antagonists have been shown to increase dopamine signaling by acting on D2 autoreceptors. Inhibition of these presynaptic receptors has been reported to enhance the release of dopamine from axon terminals, to decrease the activity of DAT, and to increase TH activity (Ford, 2014). However, when we tested if a D2-selective antagonist could be used to improve learning and memory in *Pink1*^{-/-} mice, no benefit was observed (data not shown). In contrast, administration of the dopamine D1 agonist SKF38393 was found to improve both spontaneous alternation and contextual discrimination in *Pink1*^{-/-} mice. These effects were specific to the *Pink1*^{-/-} mice as SKF38393 had either no effect or slightly impaired age-matched wild-type controls.

Previous studies have indicated that DCX-positive immature neurons in the dentate gyrus play a role in certain hippocampus-dependent behaviors, especially those that rely on pattern separation such as the context discrimination task (Glover et al., 2017; Kheirbek et al., 2012; McClelland and Goddard, 1996; Saxe et al., 2006; Treves and Rolls, 1992). As we observed that *Pink1*^{-/-} mice had difficulty performing the contextual discrimination task, it is possible that the loss of DCX-positive cells and others (Agnihotri et al., 2017) observed may have contributed to this dysfunction. It has been previously reported that dopamine promotes neurogenesis and newborn neuron survival via dopamine D1-receptors in the adult

hippocampus (Takamura et al., 2014). Thus, it is possible that the decrease in TH fibers we observed in the hippocampus may contribute to the long-term loss of adult-born neurons, and that strategies to restore dopamine signaling may also have long-term benefits by promoting newborn neuron survival. However, while our finding that treatment with a dopamine D1 agonist was sufficient to improve contextual discrimination performance, it was most likely an acute affect rather than preservation of newborn neurons as it has been estimated that newborn neurons require 4–6 weeks to be functionally incorporated into the hippocampal circuitry. Additional studies will be required to explore this and other potential long-term benefits of D1 receptor stimulation.

One caveat of these studies is that although the route of administration (i.e. systemic) has translational value, we cannot specifically attribute the effect of systemically administered SKF38393 to the hippocampus. A second caveat of this study is that all our studies were carried out using 9 to 11 month old mice. It has been previously reported that younger *Pink1*^{−/−} mice (2–6 months of age) have subtle motor problems as indicated by performance on a pole test and a cylinder test (Kelm-Nelson et al., 2018). Although we did not detect any motor dysfunction in 9 to 11 month old *Pink1*^{−/−} mice, this could possibly be due to motivational differences in the motor tasks employed between the two studies (self-initiated movements versus forced motor activity). In summary, our results suggest that loss of dopamine signaling and impaired learning and memory is a consequence of *Pink1* loss, and that D1 receptor agonists may have therapeutic benefit.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Aarsland D, Creese B, Politis M, Chaudhuri KR, Ffytche DH, Weintraub D, Ballard C, 2017 Cognitive decline in Parkinson disease. *Nat. Rev. Neurol* 13, 217–231. [PubMed: 28257128]
- Agnihotri SK, Shen R, Li J, Gao X, Bueler H, 2017 Loss of PINK1 leads to metabolic deficits in adult neural stem cells and impedes differentiation of newborn neurons in the mouse hippocampus. *FASEB J* 31, 2839–2853. [PubMed: 28325755]
- Angoa-Perez M, Kane MJ, Briggs DI, Francescutti DM, Kuhn DM, 2013 Marble burying and nestlet shredding as tests of repetitive, compulsive-like behaviors in mice. *J. Vis. Exp.* 50978. [PubMed: 24429507]
- Chomczynski P, Sacchi N, 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem* 162, 156–159. [PubMed: 2440339]
- Edelmann E, Lessmann V, 2018 Dopaminergic innervation and modulation of hippocampal networks. *Cell Tissue Res* 373, 711–727. [PubMed: 29470647]

- Ford CP, 2014 The role of D2-autoreceptors in regulating dopamine neuron activity and transmission. *Neuroscience* 282:13–22. doi: 10.1016/j.neuroscience.2014.01.025. Epub; 2014 Jan 23., 13–22. [PubMed: 24463000]
- Frankland PW, Cestari V, Filipkowski RK, McDonald RJ, Silva AJ, 1998 The dorsal hippocampus is essential for context discrimination but not for contextual conditioning. *Behav. Neurosci* 112, 863–874. [PubMed: 9733192]
- Gautier CA, Kitada T, Shen J, 2008 Loss of PINK1 causes mitochondrial functional defects and increased sensitivity to oxidative stress. *Proc. Natl. Acad. Sci. U. S. A* 105, 11364–11369. [PubMed: 18687901]
- Getz SJ, Levin B, 2017 Cognitive and Neuropsychiatric Features of Early Parkinson's Disease. *Arch Clin Neuropsychol* 32, 769–785. [PubMed: 29077803]
- Glover LR, Schoenfeld TJ, Karlsson RM, Bannerman DM, Cameron HA, 2017 Ongoing neurogenesis in the adult dentate gyrus mediates behavioral responses to ambiguous threat cues. *PLoS Biol* 15, e2001154. [PubMed: 28388632]
- Hauser DN, Primiani CT, Cookson MR, 2017 The Effects of Variants in the Parkin, PINK1, and DJ-1 Genes along with Evidence for their Pathogenicity. *Curr. Protein Pept. Sci* 18, 702–714. [PubMed: 26965687]
- Ishihara-Paul L, Hulihan MM, Kachergus J, Upmanyu R, Warren L, Amouri R, Elango R, Prinjha RK, Soto A, Kefi M, Zouari M, Sassi SB, Yahmed SB, El Euch-Fayeche G, Matthews PM, Middleton LT, Gibson RA, Hentati F, Farrer MJ, 2008 PINK1 mutations and parkinsonism. *Neurology* 71, 896–902. [PubMed: 18685134]
- Jay TM, 2003 Dopamine: a potential substrate for synaptic plasticity and memory mechanisms. *Prog. Neurobiol* 69, 375–390. [PubMed: 12880632]
- Johnson A, van der Meer MA, Redish AD, 2007 Integrating hippocampus and striatum in decision-making. *Curr. Opin. Neurobiol* 17, 692–697. [PubMed: 18313289]
- Kane LA, Lazarou M, Fogel AI, Li Y, Yamano K, Sarraf SA, Banerjee S, Youle RJ, 2014 PINK1 phosphorylates ubiquitin to activate Parkin E3 ubiquitin ligase activity. *J. Cell Biol* 205, 143–153. [PubMed: 24751536]
- Kehagia AA, Barker RA, Robbins TW, 2010 Neuropsychological and clinical heterogeneity of cognitive impairment and dementia in patients with Parkinson's disease. *Lancet Neurol* 9, 1200–1213. [PubMed: 20880750]
- Kelm-Nelson CA, Brauer AFL, Barth KJ, Lake JM, Sinnen MLK, Stehula FJ, Muslu C, Marongiu R, Kaplitt MG, Ciucci MR, 2018 Characterization of early-onset motor deficits in the Pink1^{-/-} mouse model of Parkinson disease. *Brain Res* 1680:1–12. doi: 10.1016/j.brainres.2017.12.002. Epub; 2017 Dec 8., 1–12. [PubMed: 29229503]
- Kempadoo KA, Mosharov EV, Choi SJ, Sulzer D, Kandel ER, 2016 Dopamine release from the locus coeruleus to the dorsal hippocampus promotes spatial learning and memory. *Proc. Natl. Acad. Sci. U. S. A* 113, 14835–14840. [PubMed: 27930324]
- Kheirbek MA, Tannenholz L, Hen R, 2012 NR2B-dependent plasticity of adult-born granule cells is necessary for context discrimination. *J Neurosci* 32, 8696–8702. [PubMed: 22723709]
- Kirkby RJ, Stein DG, Kimble RJ, Kimble DP, 1967 Effects of hippocampal lesions and duration of sensory input on spontaneous alternation. *J. Comp Physiol Psychol* 64, 342–345. [PubMed: 6050582]
- Kitada T, Pisani A, Porter DR, Yamaguchi H, Tscherter A, Martella G, Bonsi P, Zhang C, Pothos EN, Shen J, 2007 Impaired dopamine release and synaptic plasticity in the striatum of PINK1-deficient mice. *Proc. Natl. Acad. Sci. U. S. A* 104, 11441–11446. [PubMed: 17563363]
- Koyano F, Okatsu K, Kosako H, Tamura Y, Go E, Kimura M, Kimura Y, Tsuchiya H, Yoshihara H, Hirokawa T, Endo T, Fon EA, Trempe JF, Saeki Y, Tanaka K, Matsuda N, 2014 Ubiquitin is phosphorylated by PINK1 to activate parkin. *Nature* 510, 162–166. [PubMed: 24784582]
- Lennartz RC, 2008 The role of extramaze cues in spontaneous alternation in a plus-maze. *Learn. Behav* 36, 138–144. [PubMed: 18543713]
- Lisman JE, Grace AA, 2005 The hippocampal-VTA loop: controlling the entry of information into long-term memory. *Neuron* 46, 703–713. [PubMed: 15924857]

- Massie A, Schallier A, Vermoesen K, Arckens L, Michotte Y, 2010 Biphasic and bilateral changes in striatal VGLUT1 and 2 protein expression in hemi-Parkinson rats. *Neurochem. Int* 57, 111–118. [PubMed: 20450947]
- McClelland JL, Goddard NH, 1996 Considerations arising from a complementary learning systems perspective on hippocampus and neocortex. *Hippocampus* 6, 654–665. [PubMed: 9034852]
- McDonald C, Gordon G, Hand A, Walker RW, Fisher JM, 2018 200 Years of Parkinson's disease: what have we learnt from James Parkinson? *Age Ageing* 47, 209–214. [PubMed: 29315364]
- Papagno C, Trojano L, 2018 Cognitive and behavioral disorders in Parkinson's disease: an update. I: cognitive impairments. *Neurol Sci* 39, 215–223. [PubMed: 29043468]
- Pryde KR, Smith HL, Chau KY, Schapira AH, 2016 PINK1 disables the anti-fission machinery to segregate damaged mitochondria for mitophagy. *J. Cell Biol* 213, 163–171. [PubMed: 27091447]
- Ragozzino ME, Unick KE, Gold PE, 1996 Hippocampal acetylcholine release during memory testing in rats: augmentation by glucose. *Proc. Natl. Acad. Sci. U. S. A* 93, 4693–4698. [PubMed: 8643466]
- Rodriguez RM, Wetsel WC, 2006 Frontiers in Neuroscience Assessments of Cognitive Deficits in Mutant Mice, in: Levin ED, Buccafusco JJ (Eds.), *Animal Models of Cognitive Impairment*. CRC Press/Taylor & Francis Taylor & Francis Group, LLC., Boca Raton (FL).
- Saxe MD, Battaglia F, Wang JW, Malleret G, David DJ, Monckton JE, Garcia AD, Sofroniew MV, Kandel ER, Santarelli L, Hen R, Drew MR, 2006 Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus. *Proc Natl Acad Sci U S A* 103, 17501–17506. [PubMed: 17088541]
- Shohamy D, Adcock RA, 2010 Dopamine and adaptive memory. *Trends Cogn Sci* 14, 464–472. [PubMed: 20829095]
- Takamura N, Nakagawa S, Masuda T, Boku S, Kato A, Song N, An Y, Kitaichi Y, Inoue T, Koyama T, Kusumi I, 2014 The effect of dopamine on adult hippocampal neurogenesis. *Prog Neuropsychopharmacol Biol Psychiatry* 50, 116–124. [PubMed: 24374069]
- Torack RM, Morris JC, 1992 Tyrosine hydroxylase-like (TH) immunoreactivity in Parkinson's disease and Alzheimer's disease. *J. Neural Transm. Park Dis. Dement. Sect 4*, 165–171. [PubMed: 1349211]
- Treves A, Rolls ET, 1992 Computational constraints suggest the need for two distinct input systems to the hippocampal CA3 network. *Hippocampus* 2, 189–199. [PubMed: 1308182]
- Wang X, Winter D, Ashrafi G, Schlehe J, Wong YL, Selkoe D, Rice S, Steen J, LaVoie MJ, Schwarz TL, 2011 PINK1 and Parkin target Miro for phosphorylation and degradation to arrest mitochondrial motility. *Cell* 147, 893–906. [PubMed: 22078885]
- Wittmann BC, Schott BH, Guderian S, Frey JU, Heinze HJ, Düzel E, 2005 Reward-related fMRI activation of dopaminergic midbrain is associated with enhanced hippocampus-dependent long-term memory formation. *Neuron* 45, 459–467. [PubMed: 15694331]
- Yabe I, Yaguchi H, Kato Y, Miki Y, Takahashi H, Tanikawa S, Shirai S, Takahashi I, Kimura M, Hama Y, Matsushima M, Fujioka S, Kano T, Watanabe M, Nakagawa S, Kunieda Y, Ikeda Y, Hasegawa M, Nishihara H, Ohtsuka T, Tanaka S, Tsuboi Y, Hatakeyama S, Wakabayashi K, Sasaki H, 2018 Mutations in bassoon in individuals with familial and sporadic progressive supranuclear palsy-like syndrome. *Sci. Rep* 8, 819–19198. [PubMed: 29339765]

1. Hippocampal mitochondrial length is not affected by loss of Pink1
2. *Pink1*^{-/-} mice have reduced hippocampal tyrosine hydroxylase immunoreactivity
3. *Pink1*^{-/-} mice are impaired in hippocampus-dependent tasks compared to matched WT mice
4. Cognition in *Pink1*^{-/-} mice is improved by treatment with a dopamine D1 agonist

Fig. 1. Mitochondrial length is not altered in the hippocampi of *Pink1*^{-/-} mice.

(A) Representative TEM images showing isolated mitochondria of various sizes. (B) Summary data (binned into 0.5 μm bins) of the length distribution of 1200 randomly selected mitochondria isolated from six wild-type (WT) and six *Pink1*^{-/-} mice (200 mitochondria/animal).

Fig. 2. *Pink1*^{-/-} mice do not show overt hippocampal cell loss or altered synaptic protein levels.

(A) Representative photomicrographs of hippocampi from wild-type and *Pink1*^{-/-} mice immunostained for the neuronal marker NeuN. (B) Representative photomicrographs of calbindin D28K immunostained dentate gyri from a WT and a KO mouse. Summary data showing that the number of calbindin-D28k-positive granule neurons was not significantly different between the *Pink1*^{-/-} and WT mice (n=5/group). Data are presented as the mean ± SEM. (C) Representative photomicrographs of hippocampi from wild-type and *Pink1*^{-/-} mice immunostained for vesicular glutamate transporter 1 (vGlut1), vesicular glutamate transporter 2 (vGlut2) or Bassoon. Scale bars, 500 μm.

Fig 3. *Pink1*^{-/-} mice have normal grip strength and locomotor function.

Pink1^{-/-} mice performed as well as age-matched WT mice in the (A) wire hang and (B) rotarod tasks. (C) A representative colorized image of paw positions for an animal walking on a clear treadmill showing forepaw (FP) and hindpaw (HP) stance, and paw angle. (D) Representative walking trace (left forepaw shown) and how stride length, braking time, propelling time, and stride time are calculated. (E) Summary data comparing FP and HP gait metrics between WT and *Pink1*^{-/-} mice (n=10/group). Data are presented as the mean ± SEM. *, p<0.05.

Fig 4. Loss of Pink1 impairs hippocampal dependent behaviors.

(A) Drawing of the spontaneous alternation task. Arms were designated A-D and were surrounded by extramaze cues. Entries into the arms were recorded as the mouse explored the arena. Successful alternations (green outline) required the mouse to enter all 4 arms out of 5 entries. (B) *Pink1*^{-/-} mice made significantly fewer alternations than WT mice, suggesting hippocampal dysfunction. WT and mice made (C) similar numbers of entries and (D) traveled the same distance, indicating normal exploratory behaviors. (E) In the context discrimination task, mice are trained to differentiate between two similar chambers (pictured), based on the presence ("shock" chamber) or absence ("safe" chamber) of a footshock. (F) Wild-type mice (n=9) are able to learn to differentiate between the "shock"

and “safe” contexts as indicated by significantly more freezing behavior in the “shock” chamber. **(G)** *Pink1*^{-/-} mice (n=8) are impaired in their ability to discriminate between the two contexts, as indicated by similar percent freezing in the “shock” and “safe” chambers. **(H)** There was no significant difference in # of marbles buried in the marble burying task, a test of generalized anxiety and repetitive behaviors. **(I)** Representative tracks showing the paths taken by a WT and a KO mouse in the open field task. The center of the arena is designated by the light brown box. No significant differences were observed in either **(J)** distance traveled, **(K)** entries into the center of the arena, or **(L)** percent time spent in the center of the arena. Data are presented as the mean $\pm$ SEM. *, $p < 0.05$ by two-way ANOVA.

Fig 5. *Pink1*^{-/-} mice have reduced hippocampal tyrosine hydroxylase levels.

(A) Representative photomicrographs showing TH immunoreactivity in the dentate gyrus/hilar region of the hippocampus of wild-type (WT) and *Pink1*^{-/-} mice. Scale bar: 250 μ m.

(B) Representative westerns and **(C)** summary data (n=4/group) showing TH immunoreactivity in hippocampus (Hip), striatum (Str), and ventral tegmental area (VTA) in samples from WT and *Pink1*^{-/-} mice. Data was normalized against Actb. Please note that different amounts of starting material were used for each structure in order to ensure the TH immunoreactivity was within the linear range. As such, comparisons across structures cannot be made. **(D)** Summary data (n=4/group) showing the threshold cycle (TC) for detection of TH mRNA in hippocampus, striatum, and VTA in hippocampal samples from WT and KO mice. Data was normalized using the average threshold cycle for *Actb* and *Gapdh*. **(E)** Representative westerns and summary data (n=5/group) showing K63-linked ubiquitination on TH (immunoprecipitated with a phosphor-Ser40 TH antibody) is significantly increased in hippocampal samples obtained from KO mice compared to WT controls.

Fig 6. Proteins involved in dopamine biosynthesis and signaling are reduced in the hippocampus of *Pink1*^{-/-} mice.

(A) Simplified drawing showing the key enzymes in dopamine biosynthesis and signaling.

(B) Representative westerns and (C) summary data (n=4/group) showing the immunoreactivity of Ddc, Slc6a3, Drd2, Drd1, Dbh, Tph2, Chat and Actb in hippocampal protein samples from wild-type and *Pink1*^{-/-} animals. Actb, beta-actin; Chat, choline acetyltransferase; DA, dopamine; Dbh, dopamine beta hydroxylase; Ddc, dopa decarboxylase; Drd1, dopamine D1 receptor; Drd2, dopamine D2 receptor; KO, knock out; Slc6a3, dopamine transporter; TH, tyrosine hydroxylase; Tph2, tryptophan hydroxylase 2; WT, wild-type. Data are presented as the mean ± SEM. *, p<0.05.

Fig 7. Cognitive deficits in *Pink1*^{-/-} mice can be reversed by administration of a dopamine D1 receptor agonist.

(A) Summary data showing the performance of age-matched wild-type (WT; n=10) and *Pink1*^{-/-} (n=10) mice in the spontaneous alternation task. Pre: baseline performance; SKF38393: 30 min after 1.0 mg/kg SKF38393 administration, Washout: one week after drug administration. Performance in the contextual discrimination task of (B) *Pink1*^{-/-} mice treated with vehicle, and (C) *Pink1*^{-/-} mice treated with SKF38393. *Pink1*^{-/-} mice treated with SKF38393 showed improvement in their ability to differentiate between the “shock” and “safe” contexts. (D) age-matched WT mice treated with vehicle, (E) age-matched WT

mice treated with SKF38393. Data are presented as the mean $\pm$ SEM. *, $p < 0.05$ by two-way ANOVA.