Hyperexcitable Substantia Nigra Dopamine Neurons in \textit{PINK1}- and \textit{HtrA2/Omi}-Deficient Mice

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Bishop MW, Chakraborty S, Matthews GA, Dougalis A, Wood NW, Festenstein R, Ungless MA. Hyperexcitable substantia nigra dopamine neurons in \textit{PINK1}- and \textit{HtrA2/Omi}-deficient mice. \textit{J Neurophysiol} 104: 3009–3020, 2010. First published October 6, 2010; doi:10.1152/jn.00466.2010. The electrophysiological properties of substantia nigra pars compacta (SNC) dopamine neurons can influence their susceptibility to degeneration in toxin-based models of Parkinson’s disease (PD), suggesting that excitotoxic and/or hypoactive mechanisms may be engaged during the early stages of the disease. It is unclear, however, whether the electrophysiological properties of SNC dopamine neurons are affected by genetic susceptibility to PD. Here we show that deletion of PD-associated genes, \textit{PINK1} or \textit{HtrA2/Omi}, leads to a functional reduction in the activity of small-conductance \(
\text{Ca}^{2+}\) -activated potassium channels. This reduction causes SNC dopamine neurons to fire action potentials in an irregular pattern and enhances burst firing in brain slices and in vivo. In contrast, \textit{PINK1} deletion does not affect firing regularity in ventral tegmental area dopamine neurons or substantia nigra pars reticulata GABAergic neurons. These findings suggest that changes in SNC dopamine neuron excitability may play a role in their selective vulnerability in PD.

\textbf{INTRODUCTION}

Mitochondrial dysfunction has been widely implicated in the pathogenesis of Parkinson’s disease (PD) (Abou-Sleiman et al. 2006; Henchcliffe and Beal 2008; Schapira 2008), although how this dysfunction triggers the selective loss of substantia nigra pars compacta (SNC) dopamine neurons remains unclear. Studies on familial forms of PD have identified mutations in several genes that encode mitochondrial proteins, including \textit{PINK1}, \textit{DJ-1}, \textit{parkin}, and \textit{HtrA2/Omi} (Abou-Sleiman et al. 2006). SNC dopamine neurons, however, do not preferentially express these genes, and therefore some other factors must convey their selective vulnerability (Sulzer 2007). Of the many suggestions, one influential idea is that the unusual electrophysiological properties of dopamine neurons play a central role. For example, their unusual reliance on voltage-dependent \text{L-type Ca}^{2+} \text{ channels confers a susceptibility to mitochondrial toxins used to create animal models of PD (Chan et al. 2007). Moreover, mitochondrial toxins induce hypoactivity of SNC dopamine neurons through the activation of ATP-sensitive potassium (\text{K}_{\text{ATP}}) channels, suggesting that a loss of electrical activity can also contribute to the degeneration of these neurons (Liss et al. 2005). Indeed inactivation of either \text{L-type Ca}^{2+} \text{ channels or K}_{\text{ATP}} \text{ channels protects SNC dopamine neurons in toxin-based mouse models of PD (Chan et al. 2007; Liss et al. 2005). These studies suggest that excitotoxic and/or hypoactive mechanisms may be engaged during the early stages of PD and provide evidence of selective changes that can influence dopamine neuron survival. Consequently, we hypothesized that genetic susceptibility to PD, in particular the loss of mitochondria-associated genes, may alter the excitability of SNC dopamine neurons. Among the familial PD genes known, \textit{PTEN-induced kinase 1 (PINK1)} provides the most direct link to mitochondria. PINK1 is a serine/threonine kinase that has been shown to be localized to mitochondria both in vitro and in vivo (Gandhi et al. 2006; Muqit et al. 2006; Silvestri et al. 2005). Although the targets for PINK1 remain unknown, several PD-associated genes have been found to interact with PINK1 including the E3 ubiquitin ligase Parkin (Clark et al. 2006; Exner et al. 2007; Park et al. 2006) and the stress-protective protease HtrA2/Omi (Plun-Favreau et al. 2007). In \textit{Drosophila}, loss of \textit{PINK1} causes severe mitochondrial pathology and leads to both muscle and neuronal degeneration (Park et al. 2006; Yang et al. 2006). In mammals, studies on \textit{PINK1}-deficient mice have reported mitochondrial dysfunction in the absence of dopamine neuron loss (Gautier et al. 2008; Gispert et al. 2009; Kitada et al. 2007). Despite this, \textit{PINK1}-deficient mice display deficiencies in striatal dopamine release (Gispert et al. 2009; Kitada et al. 2007) and altered synaptic plasticity (Kitada et al. 2007) that can lead to age-dependent motor impairments (Gispert et al. 2009). We, therefore, took advantage of this mouse model to directly address whether the loss of a mitochondrial-associated PD gene can affect SNC dopamine neuron excitability. To do this, we conducted electrophysiological recordings from individual SNC dopamine neurons in ex vivo brain slice and in vivo.

\textbf{METHODS}

\textbf{Animals}

\textit{PINK1}-deficient mice were generated by Lexicon Genetics (The Woodlands, TX), and \textit{HtrA2/Omi}-deficient mice were generated by Martins et al. (2004). Generation procedures for \textit{PINK1}- and \textit{HtrA2/Omi}-deficient mice can be found here (Martins et al. 2004; Wood-Kaczmar et al. 2008). Mice were initially backcrossed on a C57BL/6 background and then interbred to generate study populations of animals containing all three possible genotypes [wild type, heterozygous, and homozygous (\textit{PINK1}-deficient)]. Experimental wild type and \textit{PINK1}-deficient mice were subsequently identified using PCR-based genotyping. Animal husbandry and experimental procedures...
were performed in full compliance with the United Kingdom Animal (Scientific Procedures) Act of 1986.

Slice preparation

Electrophysiological recordings were performed on adult PINK1 mice aged ~3–4 mo and HtrA2/Omi mice aged ~1–2 mo. To prepare SNC slices, animals were anesthetized with isoflurane and decapitated. Thin 220 μm horizontal midbrain slices were cut with a Vibratome (Leica VT1000S) while being bathed in an ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM) 120 NaCl, 25 NaHCO3, 3.5 KCl, 1.25 NaH2PO4, 2 CaCl2, 1 MgCl2, and 10 glucose, bubbled with a mixture of 95% O2-5% CO2. After sectioning, slices were allowed to recover for ≥45 min before being placed in the recording chamber and superfused with oxygenated ACSF at 31–32°C at a rate of 2–4 ml/min. Midbrain slices containing a clearly defined SNC at the level of the medial terminal nucleus (MT, medial terminal nucleus of the accessory optic tract) were used for the experiments.

In vitro electrophysiology

Cells were visualized using infrared differential interference contrast video microscopy. Conventional tight-seal (>3GΩ) whole cell patch clamp and cell-attached recordings were made using an NPI SEC-10LX amplifier (npi) and WinWCP software (Courtesy of John Dempster, University of Strathclyde) or Spike2 v5 (CED). For cell-attached recordings, electrodes (2–5 MΩ) made from borosilicate glass (Harvard Apparatus) were filled with 120 mM NaCl. For whole cell recordings, electrodes were filled with internal solution containing (in mM) 140 K-gluconate, 5 NaCl, 1 MgCl2, 10 HEPES, 2 Mg-ATP, and 0.5 Li-GTP. Neurobiotin (0.1%) was also added to the intracellular solution for labeling of recorded neurons. Records were filtered at 1 kHz and digitized at 3–5 kHz. Dopamine neurons were identified by having a spontaneous pacemaker activity of 1–4 Hz and the presence of a large L-type dependent voltage sag following injection of hyperpolarizing current. Neurochemical identity was subsequently confirmed using co-immunohistochemical-labeling for neurobiotin and tyrosine hydroxylase (see following text). Spontaneous firing activity was observed in current-clamp mode immediately after forming a whole cell configuration. \( \text{I}_{\text{leak}} \) current was assayed for in voltage-clamp by holding a voltage holding of −50 mV and applying a series of hyperpolarizing voltage steps for 1 s in −10 mV increments to −120 mV. Initial currents following the capacitive spike were used as a measure of input resistance. Linear fits of the data near resting membrane potential were then used to determine an estimate of \( \text{R}_{\text{input}} \) (Supplementary Fig. S1).

During SK pharmacology experiments, the following concentrations of drugs were used: apamin (300 nM), apamin (300 nM) to block GABAB receptors, 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]1,4-benzodiazepine-7-sulfonamide (NBQX; 5 μM) and (−)-2-amino-5-phosphonopentanoic acid (−APV; 50 μM) to block AMPA and N-methyl-d-aspartate (NMDA) receptors, respectively, A-methyl-4-carboxyphenylglycine (MCPG) (1 mM) to block metabotropic glutamate receptors (mGLURs), and haloperidol (10 μM) to block dopamine D2 receptors. For experiments examining the effects of intracellular Ca2+ release, the following drugs were used: cyclopiazonic acid (CPA, 10 μM), which depletes endoplasmic reticulum (ER) Ca2+ stores, and CGP-37157 (10 μM), which inhibits mitochondrial Na+/Ca2+ exchanger-mediated Ca2+ release. For examining neuronal excitability, NMDA (20 μM) was used. Drugs were dissolved in ACSF and applied by bath perfusion for ≥10 min before responses were measured. Apamin, 1-EBIO, CPA, and CGP-37157 were obtained from Tocris. All other drugs were obtained from Sigma Aldrich UK.

Action potential waveforms were analyzed using WinWCP (University of Strathclyde) and Spike2 v5 (CED). The average basal instantaneous firing frequency and interspike interval (ISI) were determined across 60 successive action potentials in cell-attached mode or immediately after forming whole cell configuration with the neuron. The coefficient of variation of the ISI (CV-ISI) was calculated as the ratio of the SD ISI to the mean ISI. Average action potential waveforms for each neuron were obtained using Spike2 software to obtain values for the action potential peak, threshold, peak of the afterhyperpolarization (AHP), and time-to-peak of the AHP. Any bursts were defined as beginning when two action potentials occurred within 80 ms of each other and ending when an action potential fails to occur for 160 ms. During experiments to isolate SK channel activity, neurons were voltage-clamped at −50 mV (near their resting membrane potential) and a 2 ms depolarizing step was applied to +20 mV to trigger an uncoupled action potential. This produced an outward tail current that could be inhibited by apamin (300 nM). On application of apamin, a fast transient outward current insensitive to apamin could be observed to decay within 20 ms. The apamin-sensitive current, representing SK channel conductance, peaked at ~20 ms after the test pulse, and decayed within 300 ms. Consistent with this, the tail current could be fitted with two exponentials containing a fast deactivating (<20 ms) component and a second component which decayed slower over 300 ms. Therefore we routinely calculated the integral of the outward current from 20 to 300 ms after the test pulse to assess the charge transfer representing SK channel activity. SK channel conductance was evoked in an all-or-none manner and could be abolished by TTX (1 μM). Further increasing the test pulse amplitude or prolonging the test pulse duration added a TTX-insensitive component (Supplementary Fig. S2). Reversal potentials of isolated SK currents were determined by using a depolarizing pulse to +20 mV from a holding potential of −50 mV, followed by test potentials ranging from −100 to −50 mV in 10 mV increments. Averaged amplitudes of SK currents were then plotted as a function of holding potential and reversal potential estimated.

For examination of whole cell Ca2+ currents, potassium gluconate was substituted with cesium methanesulphonate (140 mM) in the intracellular solution, and the extracellular ACSF was supplemented with tetrodotoxin (TTX, 1 μM) and tetraethylammonium (TEA, 20 mM) to block voltage-gated Na+ and K+ channels respectively. Neurons were voltage-clamped at −70 mV and a 250 ms ramp to +30 mV was applied to measure voltage-dependent Ca2+ currents. To selectively isolate low-voltage-activated T-type Ca2+ currents, nifedipine (10 μM) was added to the ACSF to block high-voltage-activated L-type Ca2+ channels, and neurons were depolarized to −40 mV from a holding potential of −100 mV. After recording baseline currents, T-type Ca2+ currents were then inhibited in some neurons with mibefradil (10 μM) by bath perfusion (10 min). Linear leakage current was not subtracted. Cells the access resistance and capacitance of which increased significantly during the course of recording (>20%) were discarded.

Statistical analysis of in vitro data

All averaged values are expressed as means ± SD. The Kolmogorov-Smirnov test and the D’Agostino-Pearson omnibus test were used to confirm normality of data sets. Data were then analyzed using either Student t-test or two-way ANOVAs with a probability level of P < 0.05 qualifying as statistically significant. When differences were found using ANOVA, Bonferroni’s post hoc test was used for multiple pairwise comparisons (Prism, Graphpad).

In vivo electrophysiology

Wild type and PINK1-deficient mice were anesthetized with urethane (2.0 g/kg iv; Sigma); supplemental doses of ketamine (20 mg/kg

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1 The online version of this article contains supplemental data.
ip; Ketaset, Willows Francis, UK) and xylazine (2 mg/kg ip; Rompun, Bayer, Germany) were given if required. Body temperature was maintained using a homeothermic heating device (Harvard Apparatus). The depth of anesthesia was assessed by testing reflexes to a hindpaw pinch. Corneal dehydration was prevented with application of Lactri-lube eye ointment (Allergan Pharmaceuticals). A craniotomy on either side of the sagittal suture was performed centered above the SNC on either side of the sagittal suture. Dura mater overlying the exposed cortex following craniotomy was removed gently by using a very fine-toothed forceps. Saline solution (0.9% wt/vol NaCl) was applied to the exposed cortex to prevent dehydration during recording.

Glass microelectrodes were lowered into the SNC using a micro-manipulator (LSS-8000 Inchworm Microdrive System, Burleigh) to a depth of 3.5–4.4 mm from the dural surface and using the following stereotaxic coordinates: anterior, −3.08 to −3.4 mm; lateral, −0.8 to −1.3 mm. Extracellular neuronal activity was monitored using the glass microelectrode in 0.5 M NaCl or physiological saline, which was broken back to give a final tip diameter of 1–2 μm and a resistance of 6–15 MΩ (in situ). Extracellular recordings were amplified (1,000 times), band-pass filtered between 0.3 and 5.0 kHz (NeuroLog System, Digitimer) and acquired with Spike2 software (version 5.08, Cambridge Electronic Design) on a PC. Electrical interference from analog signals was minimized by using HumBug (Quest Scientific, Canada). The signals were then displayed on a digital oscilloscope (Tektronics) and captured using a 1401plus A-D converter (Cambridge Electronic Design). Data were collected from neurons exhibiting broad triphasic action potentials and a spontaneous firing rate <10 Hz. SNC neurons with these properties in vivo are uniformly dopaminergic (Brown et al. 2009; Grace and Bunney 1983). Spike2 software was used to analyze data off-line. Neuronal activity was typically measured for 2–3 min each, providing the baseline firing profile of individual neurons. At the end of experiments, some mice were given a lethal overdose of anesthetic, and brain slices were examined for histological verification of the recording sites.

The baseline firing rate of each neuron and CV-ISI was quantified over a 2 min period (with band-pass filter settings of 0.3–5 kHz). Bursts were measured using the baseline recordings and were defined as beginning when two action potentials occur within 80 ms of each other and ending when an action potential fails to occur for 160 ms.

Statistical analysis of in vivo data

All averaged values are expressed as means ± SD. Statistical comparison of firing rates, CV-ISI and percentage of spikes in bursts between wild type and PINK1-deficient neurons were made by using Student t-test with a probability level of P < 0.05 qualifying as statistically significant.

Immunohistochemistry

Following in vitro recordings, brain slices were fixed with 4% paraformaldehyde in PBS, pH 7.4, for 30 min at room temperature. The fixative was removed with four washes of PBS solution. Slices were treated for 30 min with a blocking solution containing 10% normal donkey serum (Jackson Laboratories), 0.2% BSA, and 0.5% Triton-X (Sigma) for permeabilization in PBS. Primary antibody, chicken anti-tyrosine hydroxylase (1:1,000; Abcam), was applied overnight in a carrier solution consisting of 1% donkey serum, 0.2% BSA, and 0.5% Triton-X in PBS. Afterward, slices were washed four times in PBS for 10 min and then incubated with the following secondary antibodies: AlexaFluor488 goat anti-chicken IgG (1:1,000; Molecular Probes) and streptavidin-AlexaFluor555 (1:1,000; Molecular probes for 90 min at room temperature in 0.5% Triton X in PBS). Subsequently, slices were washed six times in PBS for 5 min and mounted in VectorShield Mounting Medium (Vector Laboratories). Confocal laser scanning microscopy was performed using a Leica SP confocal microscope through a ×40 or ×63 Plan-Apochromat 1.32 numerical aperture oil immersion objective. AlexaFluor 488 was excited by a 488 nm line of an Argon laser and AlexaFluor 555 by a 561 nm line of a steady state laser. To reduce spectral bleed-through, the emission filter bands for AlexaFluor 488 and AlexaFluor 555 were restricted to 498–550 and 580–640 nm, respectively. Images were taken at a resolution of 1.024 × 1.024 and processed using Leica Confocal Software (Leica Microsystems) and Adobe Photoshop CS3 (Adobe Systems).

Following in vivo experiments, animals were given a lethal dose of anesthesia then transectally perfused with 200 ml of 0.1 M phosphate-buffered saline (PBS) solution at pH 7.4 followed by 400 ml of 4% wt/vol paraformaldehyde (PFA) solution. The brain was subsequently removed and postfixed in 4% PFA. Following perfusion and fixation, the whole mouse brain was cryoprotected in 30% sucrose in phosphate-buffered saline (PBS) solution at pH 7.4 followed by 400 ml of 4% wt/vol paraformaldehyde (PFA) solution. The brain was subsequently removed and postfixed in 4% PFA. Following perfusion and fixation, the whole mouse brain was cryoprotected in 30% sucrose in PBS, embedded in optimal cutting temperature (OCT) medium, frozen in isopentane at −50°C and sectioned at 10–30 μm on a cryostat (Leica CM1800, Leica Microsystems). The floating sections were rinsed in PBS and then processed as described for in vitro experiments. Anatomical localization of recorded neurons was assessed by examining electrode tracts in combination with immunolabeling for tyrosine hydroxylase to identify SNC dopamine neurons.

RESULTS

PINK1-deficient SNC dopamine neurons display irregular firing patterns

We compared the firing activity of individual dopamine neurons within the SNC of adult (age ~3–4 mo) wild type and PINK1-deficient mice. In brain slices, SNC dopamine neurons typically display highly regular “pacemaker” firing activity at a frequency of ~1 – 4 Hz (Grace and Onn 1989). Using whole cell recordings, we found that PINK1-deficient dopamine neurons had the same action potential firing rates as wild type neurons [Fig. 1, A–C; PINK1: 2.13 ± 0.54 (SD) Hz, n = 54; wild type (WT): 1.98 ± 0.33 Hz, n = 33; NS]. Unexpectedly, however, PINK1-deficient dopamine neurons fired action potentials in a more irregular pattern as shown by the variability in ISIs between action potentials (Fig. 1D; CV-ISI, PINK1: 1.29 ± 0.19, n = 54; WT: 0.16 ± 0.10, n = 33; P < 0.05). No differences were observed in other basic properties including cell capacitance (PINK1: 29.94 ± 6.91 pF, n = 54; WT 31.24 ± 5.23 pF, n = 33; NS), input resistance (Supplementary Fig. S1; PINK1: 176 ± 65 MΩ, n = 54; WT: 161 ± 56 MΩ, n = 33; NS), and hyperpolarization-activated cation (Ih) currents (Supplementary Fig. S1). To confirm that the firing irregularity was not an artifact of the whole cell configuration, we conducted cell-attached recordings which also revealed a difference in firing regularity between PINK1-deficient and wild type dopamine neurons (Fig. 1D; CV-ISI, PINK1: 0.27 ± 0.19, n = 14; WT: 0.15 ± 0.08, n = 17; P < 0.05) without any changes in firing rate (PINK1: 1.98 ± 0.46 Hz, n = 14; WT: 2.13 ± 0.84 Hz, n = 17; NS).

PINK1-deficient SNC dopamine neurons have reduced SK channel function

Firing regularity in SNC dopamine neurons is strongly controlled by small conductance Ca2+-activated potassium channels (SK) (Wolftart and Roepfer 2002; Wolfart et al. 2001). SK channels are activated by the transient elevation of intracellular Ca2+ during an action potential, and their activity contributes to a prolonged AHP during which the return to

\[ E_{SK} = \frac{g_{SK} \cdot (V - V_{sk})}{C_{m}} \]
baseline reflects the decay of intracellular Ca\(^{2+}\) levels (Stocker 2004). We therefore tested the possibility that a reduction in SK channel function was causing firing irregularity in PINK1-deficient neurons. Consistent with this, comparison of action potential waveforms between wild type and PINK1-deficient neurons showed that the time to peak of the AHP (TTP-AHP) was selectively reduced in PINK1-deficient neurons (Fig. 1E).

Moreover, pharmacological blockade of SK channels, with apamin (300 nM), normalized the differences in firing irregularity between PINK1-deficient and wild type neurons (Fig. 2, A and B; PINK1 CV-ISI, baseline: 0.24 ± 0.05, apamin: 0.45 ± 0.13, n = 8; WT, baseline: 0.11 ± 0.02, apamin: 0.52 ± 0.15, n = 7; P < 0.05). Conversely, pharmacological facilitation of SK channel function, with 1-EBIO (200 μM), restored the firing regularity of PINK1-deficient neurons to that observed in wild type neurons (Fig. 2, C and D; CV-ISI, PINK1 baseline: 0.29 ± 0.08, n = 8; WT baseline: 0.11 ± 0.03, n = 6; P < 0.05; PINK1 1-EBIO: 0.07 ± 0.12, n = 8; WT 1-EBIO: 0.09 ± 0.03, >n = 6; NS).

These pharmacological experiments are consistent with the hypothesis that there is a functional reduction in SK channel activation in PINK1-deficient neurons. To directly examine this, we isolated SK channel-mediated currents. Neurons were voltage-clamped at −50 mV (near their resting membrane potential) and a 2 ms depolarizing step to +20 mV was used to evoke a single unclamped action potential (Supplementary Fig. S2). This protocol produced an outward tail current lasting ≤300 ms. The fast component of this current was insensitive to apamin and decayed within 20 ms. A slower apamin-sensitive SK channel-mediated component peaked at 10–20 ms (Fig. 2E). No significant differences were observed in apamin-insensitive currents between wild type and PINK1-deficient neurons (PINK1: 1.28 ± 0.47 pC, n = 33; WT: 1.37 ± 0.34 pC, n = 24; ns). Consequently, we calculated the integral of the outward tail current from 20 to 300 ms after the test pulse to assess the charge-transfer representing SK channel activity. This analysis revealed a reduction in SK channel-mediated currents in PINK1-deficient neurons compared with wild type (Fig. 2E; PINK1: 10.98 ± 2.61 pC, n = 33; WT: 16.44 ± 3.63 pC, n = 24; P < 0.05). Furthermore, increasing the open probability of SK channels using 1-EBIO restored tail current charge transfer in PINK1-deficient neurons to wild type baseline levels (Fig. 2E; charge transfer, PINK1: 15.72 ± 1.92 pC, n = 6; WT: 21.68 ± 1.48 pC, n = 5; P < 0.05). We did not observe any differences in SK channel reversal potential (WT: −87.10 ± 6.35 mV, n = 7; PINK1: −87.52 ± 9.55 mV, n = 6; P > 0.05). Moreover, when we administered longer test pulses to prolong Ca\(^{2+}\) entry into the neuron and saturate SK channel activity, we no longer observed a significant difference between PINK1-deficient and wild type neurons (Supplementary Fig. S3, A–D). This suggests that wild type levels of functional SK channels are present in PINK1-deficient neurons but are suboptimally activated during pacemaker firing. Consistent with this, using double immunolabeling for tyrosine hydroxylase (the rate-limiting enzyme in dopamine synthesis) and SK3 protein (the most common SK channel-subtype in the SNC) (Wolfart et al. 2001), we found no overt differences in channel expression between PINK1-deficient and wild type mice (Supplementary Fig. S3E).

Although our data are indicative of an intrinsic reduction of SK channel activity in PINK1-deficient neurons, we wanted to confirm this by isolating the intrinsic excitability of SNC dopamine neurons. To do this, we carried out current- and voltage-clamp recordings in ACESF containing synaptic blockers (agonists of GABA\(_{\alpha}\), GABA\(_{\beta}\), AMPA, NMDA, mGluR, and D2 receptors; see METHODS). Even in the presence of this pharmacological blockade, we still observed increased firing irregularity in PINK1-
deficient neurons compared with wild type (CV-ISI, PINK1: 0.20 ± 0.06, n = 16; WT: 0.12 ± 0.05 Hz, n = 13; P < 0.05). Similar to our previous data, we also found that PINK1-deficient and wild type neurons had identical firing rates (Hz, PINK1: 2.51 ± 0.81 Hz, n = 16; WT: 2.67 ± 0.72 Hz, n = 13; NS). Moreover, voltage-clamp recordings confirmed a functional reduction in SK channel activation was still present in PINK1-deficient neurons under these conditions (charge transfer, PINK1: 11.24 ± 2.84 pC, n = 16; WT: 15.23 ± 3.36 pC, n = 13; P < 0.05). It should also be noted that in the presence of TTX (which will eliminate action potential-dependent synaptic activity), we also observed a reduced depolarization-induced SK current in PINK1-deficient mice compared with controls using brief test pulses (Supplementary Fig. S3, C and D).

Impaired intracellular Ca²⁺ signaling underlies SK channel dysfunction

A deficit in SK channel function could be the result of either reduced Ca²⁺ influx through voltage-dependent T-type Ca²⁺ channels (Wolfart and Roeppe 2002) or impaired Ca²⁺-induced Ca²⁺ release from ER stores; this is known to provide amplification of local Ca²⁺ microdomains that facilitate SK channel activity (Bond et al. 2005; Wolfart and Roeppe 2002). To examine these possibilities, we measured whole cell Ca²⁺ currents induced by voltage ramp from −70 to +30 mV. We found no significant difference in the amplitude of total peak Ca²⁺ currents between PINK1-deficient and wild type neurons (Fig. 3A; PINK1: −593.93 ± 21.16 pA, n = 11; WT: −597.74 ± 36.88 pA, n = 9; NS). Although we would expect any change in T-type Ca²⁺ current activity to be evident in this overall Ca²⁺ current, we wanted to confirm this by selectively isolating low-voltage-activated Ca²⁺ channels. To do this, nifedipine (10 μM) was added to the ACSF to block high-voltage-activated L-type Ca²⁺ channels, and neurons were depolarized to −40 mV from a holding potential −100 mV. After recording baseline currents, the presence of T-type Ca²⁺ currents was confirmed in some neurons by inhibiting their activity with mibefradil (10 μM). We observed no difference in the activity of low-voltage-activated T-type Ca²⁺ channels between PINK1-deficient and wild type neurons (Fig. 3B; PINK1: 283.54 ± 146.54 pA, n = 11; WT: 269.42 ± 89.82 pA, n = 12; NS; mibefradil, PINK1: 79.67 ± 22.47 pA, n = 3; WT: 61.66 ± 26.31 pA, n = 3; NS). Together these results show that rapid Ca²⁺ signaling through voltage-sensitive Ca²⁺ channels is normal in PINK1-deficient neurons. Next we examined whether blockade of ER Ca²⁺ release could differentially affect SK channel activity in PINK1-deficient and wild type neurons. To do this, we bath applied CPA (10 μM) to neurons, which depletes ER Ca²⁺ stores (Seidler et al. 1989), and examined its effect on firing activity and SK channel-mediated currents. We found CPA had a smaller effect on the firing regularity of PINK1-deficient neurons compared with wild type (Fig. 3C; CV-ISI: PINK1, baseline: 0.24 ± 0.04, CPA: 0.39 ± 0.05, n = 5; WT, baseline: 0.11 ± 0.03, CPA: 0.38 ± 0.09, n = 6; P < 0.05). Moreover, AHP currents recorded in voltage-clamp showed a significantly smaller reduction in PINK1-deficient neurons following CPA treatment compared with wild type (Fig. 3E; percentage change in charge transfer in CPA, PINK1: 23 ± 9%, n = 5; WT: 56 ± 14%, n = 6; P < 0.05). Together, these results suggest that ER Ca²⁺ release-dependent regulation of SK channel activity is impaired in PINK1-deficient neurons.

Because PINK1 is predominantly localized and functions in the mitochondria (Deas et al. 2009), one crucial question is whether PINK1 deficiency and mitochondria play a role in regulating SK channel function. Mitochondria can directly influence the Ca²⁺ concentration in the cytosol of the cell by importing Ca²⁺ via the mitochondrial Ca²⁺ uniporter or transporting Ca²⁺ from the interior of the organelle into the cytosol by means of Na⁺/Ca²⁺ or H⁺/Ca²⁺ exchangers (Brini 2003). Uptake and release of Ca²⁺ from mitochondria can also directly regulate ER Ca²⁺ release. Moreover, mitochondria can sequester Ca²⁺ released from the ER as well as provide Ca²⁺ for replenishing ER stores (Brini 2003; Rizzuto et al. 1998). Importantly, impaired Na⁺/Ca²⁺ exchanger-mediated Ca²⁺ release occurs following loss of PINK1 function in dopamine neurons (Gandhi et al. 2009). We therefore examined whether
inhibition of the Na\(^+/\)Ca\(^{2+}\) exchanger with the specific inhibitor CGP-37157 could affect SK channel function in PINK1-deficient and wild type neurons. Current-clamp recordings showed that CGP-37157 (10 μM) had no significant effect on the regularity of PINK1-deficient neurons. In contrast, wild type neurons increased firing irregularity following CGP-37157 [Fig. 3D; (CV-ISI)]: PINK1, baseline: 0.22 ± 0.07, CGP: 0.24 ± 0.09, n = 12; WT, baseline: 0.14 ± 0.07, CGP: 0.20 ± 0.10, n = 14; P < 0.05]. Consistent with our current-clamp recordings, we observed a reduction in SK channel-mediated currents in wild type neurons following CGP-37157. Strikingly, this effect was largely absent in PINK1-deficient neurons (Fig. 3F; percentage change in charge transfer in CGP-37157, PINK1: 3 ± 3%, n = 6; WT: 19 ± 8%, n = 6; P < 0.05). These results show that mitochondrial Na\(^+/\)Ca\(^{2+}\) exchanger-mediated Ca\(^{2+}\) release regulates SK channel activity and is impaired in PINK1-deficient neurons.

**Selective effects of PINK1 deletion on SNC dopamine neurons**

Selective vulnerability of SNC dopamine neurons is a central feature of PD (Damier et al. 1999). Therefore one key question is whether a functional reduction in SK channel
activation is found in other neurons following PINK1 deletion. To directly address this, we examined firing activity and SK channel activity in ventral tegmental area (VTA) dopamine neurons and GABA neurons of the SNR. Both of these neuronal subgroups have been shown to express functional SK channels (Wolfart et al. 2001; Yanovsky et al. 2005).

In PINK1-deficient and wild type VTA dopamine neurons, we observed identical firing patterns, as shown by the CV-ISI (Fig. 4A; CV-ISI, PINK1: 0.29 ± 0.19, n = 10; WT: 0.28 ± 0.15, n = 13; P > 0.05). In general, we found that wild type VTA dopamine neurons showed more irregular firing patterns than the SNR. This is consistent with previous findings and is thought to be the consequence of a lower expression and dependence on SK channels in the VTA (Wolfart et al. 2001). Strikingly, however, a significant reduction in SK channel activity could still be observed in PINK1-deficient VTA dopamine neurons under voltage-clamp (Fig. 4B; charge transfer, PINK1: 8.66 ± 2.50 pC, n = 6; WT: 12.24 ± 1.22 pC, n = 8; P < 0.05).

Next we recorded the activity of SNR GABA neurons in PINK1-deficient and wild type mice. SNR GABA neurons show a distinct electrophysiological phenotype compared with dopamine neurons: fast spontaneous firing rates at ~20–30 Hz, short-duration spikes (<1 ms), and little or no I_h current (Nakanishi et al. 1987; Yanovsky et al. 2005). Unlike VTA dopamine neurons, PINK1 deficiency had no effect on firing patterns (Fig. 4C; CV-ISI, PINK1: 0.16 ± 0.02, n = 3; WT: 0.17 ± 0.01, n = 3; P > 0.05) or SK channel-mediated currents (Fig. 4D; charge transfer, PINK1, 12.72 ± 0.18 pC, n = 3; WT: 13.07 ± 0.71, n = 3; P > 0.05) in these neurons. This result is striking because SK channels are known to play a role in maintaining firing regularity of SNR GABA neurons (Atherton and Bevan 2005; Yanovsky et al. 2005). However, SNR GABA neurons express the SK2 subtype of SK channels compared with SK3 found in dopamine neurons (Yanovsky et al. 2005). Importantly, the AHP of action potentials recorded from SNR GABA neurons have also been shown to be insensitive to CPA (Yanovsky et al. 2005), indicating that SK channel function is not regulated by ER Ca^{2+} release in these neurons. This further suggests that CPA will not affect firing regularity. To test this hypothesis, we examined whether the activity of wild type SNR GABA neurons could be disrupted using CPA. Importantly, we found that SNR GABA neurons were insensitive to CPA (10 μM; Fig. 4E; CV-ISI, baseline: 0.18 ± 0.01, CPA: 0.19 ± 0.02, n = 3; P > 0.05) despite having a strong dependency on SK channels for firing regularity as shown by their sensitivity to apamin (300 nM; Fig. 4E; CV-ISI, baseline: 0.18 ± 0.01, apamin: 0.47 ± 0.14, n = 3; P < 0.05). Taken together, our results suggest that cell-type specific differences in intracellular Ca^{2+} signaling and SK channel-subtype expression may underlie the differential effects of PINK1 deletion on SNC dopamine neurons compared with VTA dopamine neurons and SNR GABA neurons.

PINK1-deficient SNC dopamine neurons display hyperexcitability in vivo

Dopamine neurons in vivo fire action potentials as single spikes or in bursts (typically doublets or triplets) (Grace and Bunney 1984a,b). Bursts are thought to have important consequences for dopamine signaling (Overton and Clark 1997; Redgrave et al. 2008; Schultz 2002), but they may increase the cytosolic Ca^{2+} burden and subsequent intracellular stress (Chan et al. 2009; Kuznetsov et al. 2006; Sulzer 2007). Burst firing is controlled through an interplay of synaptic inputs and SK channels (Blythe et al. 2007; Ji and Shepard 2006; Ji et al. 2009; Komendantov et al. 2004; Ping and Shepard 1999; Wolfart et al. 2001). We therefore hypothesized that PINK1-

![FIG. 4. Differential effect of PINK1-deficiency on VTA dopamine neurons and SNR GABA neurons.](http://jn.physiology.org/ by 10.1152/jn.00969.2010 on October 9, 2016)
deficient dopamine neurons would be more likely to burst fire compared with wild type neurons. Indeed, when we applied NMDA (a glutamate receptor agonist), we found that PINK1-deficient neurons were more likely to fire in a "bursting" manner compared with wild type neurons (Fig. 5, A and B; CV-ISI, PINK1: baseline, 0.25 ± 0.05; NMDA, 0.73 ± 0.19, n = 12; WT: baseline, 0.14 ± 0.04; NMDA, 0.26 ± 0.13; n = 14; P < 0.05). More importantly, the hyperexcitability observed in PINK1-deficient neurons could be blocked by application of the SK channel opener, 1-EBIO, confirming that SK channel facilitation can dampen excitability in these neurons and restore normal activity (Fig. 5, A and B; CV-ISI: PINK1, NMDA/1-EBIO: 0.21 ± 0.13, n = 6; NMDA/1-EBIO: 0.16 ± 0.04, n = 6; P > 0.05).

We next wanted to confirm that PINK1-deficient dopamine neurons exhibited increased endogenous burst firing in vivo. Consequently we conducted in vivo extracellular recordings from putative SNC dopamine neurons in anesthetized mice (Fig. 5, C–F). Consistent with our ex vivo findings, SNC dopamine neurons in PINK1-deficient mice and wild type mice had similar firing rates (Fig. 5D; PINK1: 3.01 ± 1.04 Hz, n = 30; WT: 3.84 ± 1.48, n = 28; P > 0.05). However, PINK1-deficient neurons had more irregular firing patterns (Fig. 5E; CV-ISI, PINK1: 1.07 ± 0.44, n = 30; WT: 0.69 ± 0.16, n = 28; P < 0.05) and exhibited increased burst firing compared with wild type neurons (Fig. 5F; percentage spikes in bursts, PINK1: 24.85 ± 13.15, n = 30; WT: 11.69 ± 8.68, n = 28; P < 0.05). Taken together these experiments show that PINK1-deficiency leads to hyperexcitability and increased burst firing in SNC dopamine neurons.

**HtrA2/omi-deficient SNC dopamine neurons display hyperexcitability ex vivo**

To broaden the scope of our findings, we examined firing activity and SK channel currents in HtrA2/omi-deficient mice.

**DISCUSSION**

We have found that deletion of PD-associated genes leads to a form of hyperexcitability in SNC dopamine neurons. In...
particular, we show that deletion of PINK1 or HtrA2/omi causes dopamine neurons to fire action potentials in an irregular manner and makes them more likely to fire bursts of action potentials. We have observed this deficit in vitro using whole cell and cell-attached recording methods and in vivo using extracellular recordings, which indicate that it is not an artifact of recording approach or experimental preparation. This irregularity occurs as a consequence of impaired intracellular Ca\(^{2+}\) signaling by ER and mitochondria, leading to a functional reduction of SK channel activation that regulates firing activity. Furthermore, we show that this deficit can be pharmacologically rescued by an SK channel facilitator, suggesting a potentially neuroprotective therapeutic target in the early stages of PD. Consistent with this possibility, SK channel facilitation with 1-EBIO has recently been shown to be neuroprotective in a 6-hydroxy-dopamine lesion mouse model of PD (Aumann et al. 2008).

It has been suggested that the selective vulnerability of dopamine neurons in PD is the consequence of multiple factors (Sulzer 2007). Recently particular emphasis has been placed on the role high Ca\(^{2+}\) plays in exacerbating cellular stress (Chan et al. 2009; Mosharov et al. 2009). Because SNC dopamine neuron activity entails relatively high Ca\(^{2+}\) fluxes, particularly through the involvement of L-type Ca\(^{2+}\) channels, it has been proposed that homeostatic Ca\(^{2+}\) stress is a key determinant in their selective vulnerability (Chan et al. 2007; Foehring et al. 2009). Indeed Ca\(^{2+}\) needs to be maintained within a tight physiological range in neurons or it can increase reactive oxygen species or, in more severe cases, trigger excitotoxicity leading to cell death (Beal 1998; Kress and Reynolds 2005; Schulz 2007). Our results suggest that genetic susceptibility may further increase the Ca\(^{2+}\) burden in SNC dopamine neurons by increasing their propensity to burst fire in vivo, a mechanism that is associated with additional Ca\(^{2+}\) loading through L-type Ca\(^{2+}\) channels and other voltage-gated channels (Deister et al. 2009; Johnson and Wu 2004; Kuznetsov et al. 2006). Consequently, a feed-forward mechanism may be established whereby Ca\(^{2+}\) influx from burst firing leads to additional mitochondrial dysfunction and cellular stress.

The proper functioning of mitochondria is critical for the normal physiology of neurons the specialized structure and function of which mean that a large amount of ATP supply is devoted to the maintenance of ion homeostasis. PINK1 deficiency has been shown to severely disrupt mitochondrial function in vitro and in Drosophila (Deas et al. 2009). In PINK1-deficient mice, however, mitochondrial functional defects have been shown to be relatively subtle and fail to create a major energy crisis (Gautier et al. 2008). Therefore reduced ATP levels are unlikely to be the cause of the electrophysiological deficits found in our study. A recent report has demonstrated that a loss of PINK1 affects the capacity of the mitochondrial Na\(^+\)/Ca\(^{2+}\) exchanger to release Ca\(^{2+}\), leading to mitochondrial Ca\(^{2+}\) overload. More importantly, this deficit appears to precede respiratory chain dysfunction, increased production of ROS, and, ultimately, cell death (Gandhi et al. 2009). Consistent with this, our results suggest that there is a functional reduction in SK channel activation, in part, because of a failure of mitochondrial Ca\(^{2+}\) release via the Na\(^+\)/Ca\(^{2+}\) exchanger. The milder phenotype found in PINK1-deficient mice may therefore reflect the early stages of PINK1 deficiency observed in cell models. Although it is still possible that impaired ER and mitochondrial Ca\(^{2+}\) release is a result of impaired respiration and ATP production, compromised ATP levels would be expected to activate K\(_{ATP}\) channels in SNC dopamine neurons and cause hypoactivity, as has been observed following mitochondrial inhibition in toxin-based PD mouse models (Liss et al. 2005). Thus the hyperexcitability observed in our study may represent a distinct pathophysiological mechanism that can be triggered through mitochondrial dysfunction and provides an insight into how genetic factors may modulate susceptibility in PD.

Recent studies have sparked renewed appreciation for the remarkably dynamic nature of mitochondria, particularly in the context of PINK1 and PD (Deng et al. 2008; Poole et al. 2008). Mitochondria constantly fuse and divide and are actively transported to specific subcellular locations (Karbowiak and Youle 2003; Yaffe 1999). The dynamic nature of these organelles will undoubtedly be important in their role as regulators of Ca\(^{2+}\) signaling. Although mitochondria in PINK1-deficient mice appear to be structurally intact and preserved in total number, there is a selective increase in larger mitochondria, which would be consistent with a role of PINK1 in the promotion of
mitochondrial fission (Gautier et al. 2008). Therefore it is possible that a deficit in mitochondrial Ca\(^{2+}\) flux in our study is a result of impaired mitochondrial dynamics. It will be interesting to examine whether mitochondrial trafficking and cellular distribution are also impaired in PINK1-deficient mice because mitochondrial motility is regulated by intracellular Ca\(^{2+}\) (Wang and Schwarz 2009). In addition, the kinase activity of PINK1 could directly or indirectly modulate the Na\(^+/Ca^{2+}\) exchanger and/or other mitochondrial ion channels.

It is not yet clear how mitochondrial Ca\(^{2+}\) signaling can affect SK channel function. However, mitochondria can form close contacts with the ER (de Brito and Scorrano 2008; Rizzuto et al. 1998, 2009) and the plasma membrane (Malli et al. 2003) with the ER forming a continuous intracellular network in SNC dopamine neurons that can extend throughout the somatodendritic tree (Choi et al. 2006; Schwyn and Fox 1974). The ability of mitochondria to uptake and release Ca\(^{2+}\) allows them to operate as a dynamic and reversible Ca\(^{2+}\) storage compartment that can modulate ER Ca\(^{2+}\) stores and influence the spatiotemporal pattern of intracellular Ca\(^{2+}\) signals (Brini 2003; Castaldo et al. 2009). Given the high buffering capacity of mitochondria, it is possible that PINK1 deficiency triggers mitochondrial Ca\(^{2+}\) overload similar to that observed in other studies when the Na\(^+/Ca^{2+}\) exchanger is impaired (Gandhi et al. 2009). In turn, this may suppress local ER Ca\(^{2+}\) signaling or impair reloading of ER Ca\(^{2+}\) stores, diminishing the Ca\(^{2+}\) microdomain that facilitates SK channel activation. Thus voltage-gated Ca\(^{2+}\) channels, along with ER and mitochondrial Ca\(^{2+}\) stores, may collectively regulate the Ca\(^{2+}\) microdomain that shapes SK channel activity in dopamine neurons.

Although the precise Ca\(^{2+}\) dynamics that regulate SK channel function are beyond the scope of this study, the intrinsic pacemaker activity of SNC dopamine neurons may be particularly prone to disruption during mitochondrial impairment. This is suggested by the lack of SK channel dysfunction in SNR GABA neurons, where SK channels play a similar role in maintaining firing regularity but have no reliance on intracellular Ca\(^{2+}\) release to facilitate their activity (Atherton and Bevan 2005; Yanovsky et al. 2005). In addition, using prolonged depolarizing steps to elevate intracellular Ca\(^{2+}\) levels (beyond that achieved during single action potentials) can restore SK channel activity in PINK1-deficient SNC dopamine neurons, suggesting that the deficit is specific to the temporal and spatial dynamics of tonic firing activity. VTA dopamine neurons, which are also spontaneously active, have a similar functional reduction in SK channel activation to SNC neurons. However, these neurons have less reliance on SK channels for maintaining firing regularity (Wolfart et al. 2001), which could explain the differential effects of PINK1 deficiency on the firing activity of SNC and VTA dopamine neurons.

The selective effect of PINK1 deficiency on SNC dopamine neurons has important implications toward understanding their vulnerability in PD. Although no neurodegeneration occurs in PINK1-deficient mice, it is likely that genetic mouse models require secondary “hits” to trigger neurodegeneration. This is suggested by the fact that PINK1-deficient mice show enhanced sensitivity to oxidative stressors such as H\(_2\)O\(_2\) and mild heat shock (Gautier et al. 2008). Similarly, Parkin-deficient mice have increased vulnerability to inflammatory stimuli, suggesting that an environmental trigger is sufficient to induce neurodegeneration (Frank-Cannon et al. 2008). Therefore in future studies, it will be important to examine whether SNC dopamine neurons from PINK1-deficient mice show a greater sensitivity to Ca\(^{2+}\)-induced cell death or other cellular stressors.

Here, we propose that PINK1 and HtrA2/omi deficiency leads to a feed-forward interaction between mitochondrial function and electrophysiological activity that contributes to the vulnerability of SNC dopamine neurons in PD. Because mitochondrial dysfunction is also implicated in sporadic forms of PD (Abou-Sleiman et al. 2006), changes in dopamine neuron excitability may be a common pathophysiological mechanism preceding neurodegeneration.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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