PINK1-mediated Drp1S616 phosphorylation modulates synaptic development and plasticity via promoting mitochondrial fission

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Dynamic change of mitochondrial morphology and distribution along neuronal branches are essential for neural circuitry formation and synaptic efficacy. However, the underlying mechanism remains elusive. We show here that PINK1 knockout (KO) mice display defective dendritic spine maturation, reduced axonal synaptic vesicles, abnormal synaptic connection, and attenuated long-term synaptic potentiation (LTP). Drp1 activation via S616 phosphorylation rescues deficits of spine maturation in PINK1 KO neurons. Notably, mice harboring a knockin (KI) phosphor-null Drp1S616A recapitulate spine immaturity and synaptic abnormality identified in PINK1 KO mice. Chemical LTP (cLTP) induces Drp1S616 phosphorylation in a PINK1-dependent manner. Moreover, phosphor-mimetic Drp1S616D restores reduced dendritic spine localization of mitochondria in PINK1 KO neurons. Together, this study provides the first in vivo evidence of functional regulation of Drp1 by phosphorylation and suggests that PINK1-Drp1S616 phosphorylation coupling is essential for convergence between mitochondrial dynamics and neural circuitry formation and refinement.

INTRODUCTION

Neurons are highly polarized cells that consist of numerous branches including dendrites and axons. The distribution and transportation of organelles along neural branches are of great importance for neuronal development and function. Mitochondria are vital organelles asymmetrically distributed in neuronal compartments. They are under dynamic morphological change and active trafficking during neuron development or in response to neuron activity. Mitochondrial dynamics, including morphological change and distribution, have been recognized as crucial mechanisms to support their functions in regulating energy supply, Ca²⁺ homeostasis, neurotransmitter synthesis, ROS generation, apoptosis, metabolism, and other aspects of neuron physiology. These dynamic modifications also provide a capability for spatiotemporal regulation of local cellular events to adapt to the extreme complexity of neuronal compartments including axons, dendrites, and synapses.

Mitochondrial morphology and functions are regulated by constant fusion and fission dynamics and motility. Fusion is triggered by several GTPases, for example, Mfn1 and Mfn2 at the outer membrane, and Opa1 at the inner membrane. Another GTPase in the dynamin family, Drp1 (dynamin-related protein-1) is a key component to execute fission at the outer membrane. Drp1 is recruited to the mitochondria membrane by Fis1 and MFF to facilitate fission. Notably, posttranslational modifications to Drp1 through phosphorylation, SUMOylation, or S-nitrosylation are important for its fission activity in vitro. Meanwhile, trafficking and anchoring of mitochondria are regulated by microtubule or actin-based proteins. Together, these molecular machineries coordinate to determine the size, content, shape, and localization of mitochondria, therefore are crucial for proper mitochondrial function in neurons.

Synapse is the key contact site between neurons. Numerous molecular and cellular processes consuming energy occur locally at or close to synapses. Mitochondrial dynamics is critical at these sites to support the wiring of synaptic circuitry and synaptic plasticity. Accumulating evidence suggests that alteration of mitochondrial size or disruption of key fission molecule Drp1 result in abnormalities of synaptic development, plasticity, and function. For instance, deletion of Drp1 in mice leads to aggerated mitochondria and reduced expression of synaptophysin. Drp1 knockout in forebrain excitatory neuron results in enlargement of

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mitochondria and decreased mitochondrial-derived ATP in the axon, impaired synaptic transmission, and hippocampus-dependent memory.\textsuperscript{9} Drp1 knockout in D1-Medium spiny neuron in nucleus accumbens increases the number of large mitochondria and blocks cocaine-seeking after drug exposure.\textsuperscript{10} Moreover, expression of Drp1-K38A, a dominant-negative form of Drp1, in cultured hippocampal neurons decreases mitochondrial content and dendritic spine numbers and blocks neuron activity-induced increase of postsynaptic puncta.\textsuperscript{5} In hippocampal neurons, activity-dependent enhancement of mitochondrial motility and entering into dendritic protrusions are promoted by Drp1 overexpression while inhibited by Drp1-K38A, suggesting profound synaptic influences of Drp1-mediated mitochondrial dynamics.\textsuperscript{5}

The fission activity of Drp1 is regulated by different posttranslational modifications.\textsuperscript{21,22} Drp1 phosphorylation at different serine sites, such as 616, 637, 656, plays important role in mitochondrial dynamics.\textsuperscript{12–15} Despite several in vitro studies implicating important roles of Drp1 phosphorylation in synapse development and plasticity,\textsuperscript{6,14} they are yet to be verified in vivo.

PTEN-induced kinase 1 (PINK1) encodes a serine/threonine kinase targeted to mitochondria.\textsuperscript{16} PINK1 promotes mitochondrial fission in flies.\textsuperscript{17–20} In mammalian cells, PINK1 has variable effects on fission in a different cell context.\textsuperscript{21,22} We recently demonstrated that PINK1 directly phosphorylates Drp1\textsuperscript{S616} to regulate mitochondrial fission.\textsuperscript{23}

In this study, we aimed to investigate the roles of PINK1-mediated Drp1\textsuperscript{S616} phosphorylation in the central nervous system. Our results demonstrate increased detection of elongated mitochondria in neurons in the hippocampus and cortex of Pink1\textsuperscript{KO} mice. In these mice, mitochondria are less frequently found in dendritic spines and presynaptic boutons during excitatory synapse maturation. Localization of mitochondria in dendritic spines upon LTP induction is suppressed in Pink1\textsuperscript{KO} neurons. Drp1\textsuperscript{S616}A KI mice, bearing phosphor-null mutation of Drp1, phenocopy Pink1\textsuperscript{KO} mice in defects of synaptic development and function. Notably, Drp1\textsuperscript{S616} KI mice exhibit inhibited hippocampal-dependent learning and memory. Together, our results suggest that PINK1-mediated Drp1\textsuperscript{S616} phosphorylation plays an essential role in regulating mitochondrial dynamics, therefore, contributing to synapse maturation, synaptic transmission and plasticity, and learning and memory. This study uncovered a novel kinase/substrate cassette that couples mitochondrial dynamics with the development and function of neural circuitry.

RESULTS

PINK1 regulates dendritic spine morphogenesis and excitatory synapse formation

To investigate the physiological function of PINK1 on mitochondrial dynamics during synapse development, we labeled mitochondria and cultured neurons derived from Pink1\textsuperscript{KO} and wild-type (WT) mice with MitoGFP and DsRed, respectively. Length and number of dendritic mitochondria at days in vitro (DIV) 18, a critical period of synapse maturation, were examined and quantified. The mean mitochondrial length was 3.783 ± 0.215 µm in WT hippocampal neurons, while it increased to 10.48 ± 0.647 µm in Pink1\textsuperscript{KO} neurons. In contrast, the density of dendritic mitochondria was decreased in Pink1\textsuperscript{KO} neurons (Supplementary Fig. S1a, b; 16.86 ± 0.744 mitochondria per 100 µm in WT neurons; 9.15 ± 0.452 mitochondria per 100 µm in Pink1\textsuperscript{KO} neurons). Likewise, the length of axonal mitochondria was also longer in Pink1\textsuperscript{KO} neurons than that in WT neurons (Supplementary Fig. S1a, b). Results indicate that mitochondria are longer in size but less in number in Pink1\textsuperscript{KO} neurons than those in WT neurons. Next, neuronal mitochondrial in the hippocampus and cortex were analyzed in vivo using transmission electron microscopy (TEM). Length of dendritic mitochondria increased ~1.5-fold in Pink1\textsuperscript{KO} neurons at age of 8 weeks compared to that in WT neurons (Supplementary Fig. S1c, d; 0.87 ± 0.050 µm in WT hippocampal neurons; 1.28 ± 0.100 µm in Pink1\textsuperscript{KO} hippocampal neurons. Supplementary Fig. S1e, f; 0.92 ± 0.071 µm in WT cortical neurons; 1.67 ± 0.144 µm in Pink1\textsuperscript{KO} cortical neurons). Interestingly, mitochondria were visualized in ~16.75% presynaptic boutons in WT neurons, while only in ~11.75% in Pink1\textsuperscript{KO} neurons (Supplementary Fig. S1g, h). 88.94% of WT boutons had mitochondria with a length less than 0.5 µm. In contrast, Pink1\textsuperscript{KO} boutons showed an increased number of large mitochondria (0.5–1 µm in length) compared to WT boutons (Supplementary Fig. S1g, h, ~25.69% in Pink1\textsuperscript{KO} neurons, ~11.06% in WT neurons). Together, dendritic and axonal mitochondrial fission are defective in Pink1\textsuperscript{KO} neurons.

We next studied the roles of PINK1 in the development of dendritic spines. Cortical or hippocampal neuronal cultures derived from WT or Pink1\textsuperscript{KO} mice were quantified for spine number of secondary dendrites at DIV 18. Dendritic spines are classified into three groups based on their morphology, including mushroom, thin, and stubby spines.\textsuperscript{24} Both mushroom and thin spines have enlarged heads and constricted necks that are difficult to distinguish, we, therefore, combine them into a mushroom/thin spine group in this study. Results revealed that the density of total spines was significantly lower in Pink1\textsuperscript{KO} cortical neurons than that in WT control neurons at DIV 18 (Fig. 1a, b, 75.00 ± 2.145 spines/100 µm in WT neurons; 58.07 ± 1.772 spines/100 µm in Pink1\textsuperscript{KO} neurons). Notably, a decrease in density of mushroom/thin spines (Fig. 1a, b, 67.27 ± 1.989 spines/100 µm in WT neurons; 50.96 ± 1.62 spines/100 µm in Pink1\textsuperscript{KO} neurons), but not stubby spines (Fig. 1a, b, 7.73 ± 0.401 spines/100 µm in WT neurons; 7.11 ± 0.312 spines/100 µm in Pink1\textsuperscript{KO} neurons) was observed in Pink1\textsuperscript{KO} cortical neurons compared to that of WT control neurons. In contrast, the density of filopodium was increased in Pink1\textsuperscript{KO} neurons (Fig. 1a, b, 3.62 ± 0.253 protrusions/100 µm in WT neurons; 9.65 ± 0.451 protrusions/100 µm in Pink1\textsuperscript{KO} neurons). Results indicate immaturity of spines of cortical neurons derived from Pink1\textsuperscript{KO} mice.\textsuperscript{25,26} Consistently, immature spines were observed in cultured Pink1\textsuperscript{KO} hippocampal neurons (Supplementary Fig. S2a, b). Quantification of excitatory synapses by examining colocalized Homer1, a protein enriched in the postsynaptic density of excitatory synapse, and presynaptic Synapsin1 revealed that synapse density in cultured Pink1\textsuperscript{KO} cortical neurons was lower than that in WT control neurons at DIV 18 (Fig. 1c, d). The fluorescence intensity of postsynaptic protein Homer1 was reduced in Pink1\textsuperscript{KO} dendrites compared to WT dendrites, suggesting a decreased level of Homer1 expression at synapses (Fig. 1d). However, dendritic length and number of intersections were not changed in Pink1\textsuperscript{KO} neurons compared to WT neurons (Supplementary Fig. S3a, b). Thus, PINK1 selectively regulates dendritic spine morphogenesis and excitatory synapse formation.

Pink1\textsuperscript{KO} mice exhibit abnormal hippocampal and cortical circuits

We next examined whether PINK1 regulates neuronal connections in intact brains in vivo. Golgi staining of cortical slices at postnatal day 14 (P14) showed that density of spines and mushroom/thin spines at secondary apical dendrites of layer 2/3 neurons in somatosensory cortex was reduced in Pink1\textsuperscript{KO} mice (57.09 ± 3.210 total spines/100 µm; 51.81 ± 3.003 mushroom/thin spines/100 µm) compared to that of their WT littermates (70.51 ± 3.152 total spines/100 µm; 65.39 ± 2.966 mushroom/thin spines/100 µm). By contrast, the density of filopodium was increased in Pink1\textsuperscript{KO} neurons (5.96 ± 0.394 protrusions/100 µm) compared to that of WT neurons (2.48 ± 0.232 protrusions/100 µm). However, the density of stubby spines in neurons remained similar in Pink1\textsuperscript{KO} and WT mice (Fig. 2a, b). Synapse density in hippocampal CA1 stratum radiatum and somatosensory cortex Layer 2/3, quantified by colocalization of pre- and post-synaptic puncta (Synaptophysin and PSD95), revealed a significant lower synapse density in both hippocampus and somatosensory cortex of Pink1\textsuperscript{KO} mice.
KO mice than that of WT littermates at age of 8 weeks (Fig. 2c, d). TEM analysis on a hippocampal or cortical slice of 8-week-old mice showed smaller postsynaptic density (PSD) in Pink1 KO neurons than that of WT neurons (Fig. 2e, f). Notably, the presynaptic bouton in Pink1 KO neurons was smaller and contained less synaptic vesicles compared to that in WT neurons (Fig. 2g, h, bouton size: 0.3625 ± 0.018 µm² in WT neurons, 0.3038 ± 0.014 µm² in Pink1 KO neurons; number of synaptic vesicles per bouton: 52.74 ± 2.323 in WT neurons; 41.52 ± 1.731 in Pink1 KO neurons). Results suggest defective presynaptic assembly in Pink1 KO neurons. Consistently, immunoblot analysis of crude synaptosome (P2 fraction) extracted from cortical tissues showed that PSD95 was reduced in Pink1 KO mice (Fig. 2i, j, Supplementary Fig. S2c, d). Paired-pulse facilitation (PPF) analysis of somatosensory cortex layer 2/3 neurons showed increased PPF in Pink1 KO mice compared to that in WT mice, indicating a lower probability of releasing synaptic vesicles (Supplementary Fig. S3e, f). Results establish a physiological role of PINK1 in excitatory neuronal connectivity in hippocampal and cortical circuits.

Next, we examined whether PINK1 regulates LTP induced by theta-burst stimulation (TBS) of the Schaffer collateral pathway in acute hippocampal slices. The field excitatory postsynaptic potentials (fEPSPs) recorded in Pink1 KO Schaffer collateral-CA1 synapses was greatly reduced at both initial (0–10 min after TBS, Pink1 KO 130.2% of baseline, WT 147.4% of baseline) and maintenance phase (40–60 min after TBS, Pink1 KO 117.1% of baseline, WT 140.9% of baseline) post-TBS induction (Fig. 3). Therefore, PINK1 is required for not only induction but also maintenance of LTP.
PINK1 regulates synapse maturation through Drp1<sup>S616</sup> phosphorylation.
We have recently demonstrated that PINK1 directly phosphorylates Drp1<sup>S616</sup>. Drp1 promotes synapse formation by affecting mitochondrial fission.<sup>5,8</sup> To explore the mechanism of PINK1 in regulating synapse maturation and plasticity, we looked into PINK1 substrate Drp1<sup>S616</sup> that control mitochondrial dynamics. Phosphor-Drp1<sup>S616</sup> levels were greatly reduced in hippocampal...
PINK1-mediated Drp1S616 phosphorylation regulates presynaptic exocytosis. Exocytosed synaptic vesicles in cultured neurons were labeled by an antibody against the luminal domain of the synaptic vesicle protein synaptotagmin 1. Results showed that the density of recycled synaptotagmin 1 puncta axons was decreased in Drp1S616A(KI) neurons (Fig. 4n, o), while the co-immunostained vGlut1 puncta density was comparable in Drp1S616A WT and WT neurons (Fig. 4n, o). Furthermore, PPF was increased in Drp1S616A(KI) mice compared with WT mice (Supplementary Fig. 3e, f). Results suggest that Drp1S616A phosphorylation is required for the establishment of functional presynaptic terminals that are undergoing presynaptic exocytosis.

We next determined whether PINK1 regulates synapse maturation via Drp1S616A phosphorylation. PINK1 KO neurons were co-expressing EGFP with either Drp1S616D (a phosphor-mimetic variant of Drp1), Drp1S616A (a phosphor-null variant of Drp1), or Drp1WT. As a control, neurons derived from WT control mice were co-transfected with empty control plasmid and EGFP plasmid. The numbers and morphology of dendritic spines in each group were quantified at DIV 18. PINK1 KO neurons expressing Drp1S616D or Drp1WT, but not Drp1S616A, resulted in a significant increase of density of total spines (Fig. 4p, q, 70.16 ± 1.918 spines/100 µm in PINK1 KO neurons expressing Drp1S616D or Drp1WT, 50.53 ± 2.787 spines/100 µm in PINK1 KO neurons expressing Drp1WT, 4.83 ± 0.407 protrusions/100 µm in PINK1 KO neurons expressing Drp1WT, and 76.49 ± 2.322 spines/100 µm in PINK1 KO neurons expressing Drp1S616D), and mushroom/thin spines (Fig. 4p, q, 62.97 ± 1.854 spines/100 µm in PINK1 KO neurons overexpressed Drp1WT, 50.53 ± 2.787 spines/100 µm in PINK1 KO neurons overexpressed Drp1S616D, and 68.62 ± 2.161 spines/100 µm in PINK1 KO neurons overexpressed Drp1S616D) compared to PINK1 KO neurons transfected with control empty plasmid (Fig. 4p, q, 65.07 ± 1.702 total spines/100 µm; 56.97 ± 1.608 mushroom/thin spines/100 µm). In contrast, filopodium density was decreased in PINK1 KO neurons expressing Drp1S616D or Drp1WT, but not the neurons expressing Drp1S616A (Fig. 4p, q, 5.67 ± 0.393 protrusions/100 µm in PINK1 KO neurons expressing Drp1WT, 11.09 ± 0.665 protrusions/100 µm in PINK1 KO neurons expressing Drp1WT, 4.83 ± 0.407 protrusions/100 µm in PINK1 KO neurons expressing Drp1S616D, and 76.49 ± 2.322 spines/100 µm in PINK1 KO neurons expressing Drp1S616D), and mushroom/thin spines (Fig. 4p, q, 62.97 ± 1.854 spines/100 µm in PINK1 KO neurons overexpressed Drp1WT, 50.53 ± 2.787 spines/100 µm in PINK1 KO neurons overexpressed Drp1S616D, and 68.62 ± 2.161 spines/100 µm in PINK1 KO neurons overexpressed Drp1S616D) compared to PINK1 KO neurons transfected with control empty plasmid (Fig. 4p, q, 65.07 ± 1.702 total spines/100 µm; 56.97 ± 1.608 mushroom/thin spines/100 µm).
restored to comparable levels of WT neurons, Drp1S616 phosphorylation is downstream of PINK1 in regulating spine maturation.

**PINK1-dependent Drp1S616 phosphorylation is critical for LTP induction and maintenance**

To further explore the physiological role of Drp1S616 phosphorylation in LTP, we induced LTP in Schafer collateral pathway at acute hippocampal slices prepared from Drp1S616A KO mice and their WT control littermates by TBS stimulation. Consistent with findings from Pink1 KO mice, fEPSPs recorded at CA1 were attenuated in neurons of Drp1S616A.KO mice compared to that in neurons of WT control mice at both initiation (0–10 min after TBS, Drp1S616A.KO 129.5 ± 3.479% of baseline; WT 143.8 ± 4.547% of baseline) and maintenance phase (40–60 min post-TBS, Drp1S616A.KO 108 ± 3.495% of baseline; WT 142.7 ± 6.189% of baseline) of LTP (Fig. 5a, b). Thus, Drp1S616 phosphorylation plays an essential role in LTP induction and maintenance.

We next examined Drp1S616 phosphorylation following LTP-induction by employing a tetraethylammonium chloride (TEA)-induced protocol in primary cultured neurons.27–29 The total Drp1 remained unchanged at all time points examined after 25 mM TEA treatment for 15 min (Fig. 5c, d). Notably, a rapid increase of Drp1S616 phosphorylation peaked at 2.5 min post TEA treatment (Fig. 5c, d; 216.1 ± 29.4% of baseline, p < 0.01). Phospho-Drp1S616 remains increase at 15 min after TEA administration, although not reaching statistical significance (Fig. 5c, d; 210.6 ± 20.7% of baseline, p = 0.073). In contrast, TEA did not induce Drp1S616 phosphorylation in Pink1 KO neurons (Fig. 5c, d). Moreover, phosphorylated Drp1S616 was accumulated on mitochondria after cLTP treatment, which is consistent with a previous report that Drp1S616 is required for cLTP fusion burst5 (Fig. 5e, f). Results suggest that PINK1 phosphorylates Drp1S616 upon LTP induction, thereby contributing to LTP induction and maintenance.

**PINK1-mediated Drp1S616 phosphorylation regulates mitochondrial fission during synapse development**

We next examined whether PINK1 regulates synapse morphogenesis and maturation via promoting Drp1S616 phosphorylation-mediated mitochondrial fission in neurons. Cortical neuronal cultures derived from Drp1S616A.KO and their WT control littermates were co-expressed with MitoDsRed (to label mitochondria) and EGFP (to visualize neuronal morphology). Drp1S616A.KO neurons displayed fewer but enlarged dendritic and axonal mitochondria at DIV 18 compared to those of WT control neurons, which is consistent with the observation in Pink1 KO neurons (Fig. 6a, b). TEM analysis showed that dendritic mitochondrial size is larger in Drp1S616A.KO hippocampal and cortical neurons than that in WT control neurons at the age of 8 weeks (Fig. 6c, d). In addition, presynaptic boutons in Drp1S616A.KO neurons were less frequent to contain mitochondria, although the large-sized mitochondria (0.5–1 μm in length) were observed within presynaptic boutons of Drp1S616A.KO neurons (Fig. 6e, f). Thus, Drp1S616 phosphorylation plays vital role in mitochondrial fission and distribution in neurons.

Next, we examined whether Drp1S616 phosphorylation is involved in PINK1-mediated mitochondrial fission. Primary neuronal cultures derived from Pink1 KO mice at DIV 6–8 were co-expressed MitoDsRed and EGFP with either Drp1S616A.KO, Drp1S616D, or Drp1WT. Expression of Drp1S616D or Drp1WT restored dendritic mitochondrial fission abnormality in Pink1 KO neurons (Fig. 6g, h). Expression of Drp1S616D resulted in a reduced length (3.30 ± 0.169 μm) and an increased density of mitochondria (18.66 ± 0.8014 mitochondria/100 μm) in Pink1 KO neurons compared to that of control neurons (6.652 ± 0.492 μm in length, 13.11 ± 0.617 mitochondria/100 μm). Expression of Drp1WT partially rescued mitochondria fission defect in Pink1 KO neurons (Fig. 6g, h). It is likely that other kinases for Drp1S616 exist as shown in Drosophila.23 Together, results indicate that phospho-Drp1S616 mediates PINK1 effects on mitochondrial fission during synapse development.

To test whether PINK1 affects local ATP homeostasis in dendrites, PercevalHR, a genetically encoded fluorescent ATP sensor,30 was expressed in cultured Pink1 KO and WT cortical neurons. The fluorescence intensity ratio of F488nm/F405nm, which indicates the intracellular ATP/ADP ratio, was significantly lower in Pink1 KO dendrites than that in WT dendrites (Supplementary Fig. S5a, b). Results suggest that defects of mitochondria fission in Pink1 KO neurons reduces dendritic energy supply. Meanwhile, cortical neurons (DIV 18) derived from Pink1 KO mice treated with 1 mM piracetam, a chemical to improve mitochondrial function and increase ATP production,31 partially rescued spine defects of Pink1 KO neurons (Supplementary Fig. S6a, b). Thus, PINK1/phospho-Drp1S616 regulates mitochondrial dynamics and ATP production to modulate spine maturation.

**PINK1-mediated Drp1S616 phosphorylation regulates synaptic localization and invasion of mitochondria**

At DIV 18, around 8% of dendritic protrusions contained mitochondria in wild-type control neurons. However, dendritic
protrusions with mitochondria were reduced to around 6% in Pink1 KO cortical neurons (Fig. 7a, b, basal level). Notably, this reduction of mitochondria in dendritic protrusions was rescued by expressing Drp1S616D. Results suggest that PINK1 increases mitochondrial incursion into dendritic protrusions to promote synapse/spine maturation via promoting Drp1S616 phosphorylation-mediated mitochondrial fission.

We next test whether synaptic redistribution of mitochondria occurs upon LTP induction via the PINK1/phosphor-Drp1S616 pathway. Cortical neurons (DIV 18) labeled by DsRed and MitoGFP
were treated with TEA (25 mM) for 15 min followed by fixed neurons 30 min posttreatment. Compared to the basal level, TEA treatment resulted in a ~35% decrease in dendritic protrusions containing mitochondria (Fig. 7a, b, 8.48 ± 0.531% in basal level, 11.52 ± 0.645% in TEA treatment). In contrast, TEA treatment elicited little change of dendritic protrusions containing mitochondria in Pink1 KO neurons (6.662 ± 0.3822% in basal level, 7.477 ± 0.450% in TEA treatment). Expression of Drp1S616A restored mitochondrial invasion into protrusions in Pink1 KO neurons (8.91 ± 0.642% in basal level, 11.06 ± 0.712% in TEA treatment). Moreover, TEA-induced mitochondrial fragmentation was abolished in Pink1 KO neurons that was rescued by introducing Drp1S616A (Fig. 7c). Results suggest that Pink1-mediated Drp1S616A phosphorylation regulates TEA-induced mitochondrial invasiveness into synaptic compartments.

Drp1S616A KI mice exhibited impaired hippocampal-dependent memory

The appearance and gross motor behavior of Drp1S616A KI mice were normal at age of 5 months (data not shown). We next explored the roles of phosphoryl-Drp1S616A in learning and memory by employing Morris water maze (MMW) task tests. 7-month-old mice were trained for swimming tests for 2 sessions/day for 5 days. In the first session, Drp1S616A KI mice and their wild-type control littermates were injected with DsRed followed by immunodetection of synaptotagmin 1 (Syt1) and vGlut1 at DIV 18. Representative images of Syt1-positive puncta (green, Syt1), vGlut1-positive puncta (gray, vGlut1), and axon (red, DsRed) were shown. Imaris software processed Syt1 staining (green, Imaris Syt1) and Imaris software processed vGlut1 staining (white, Imaris vGlut1) on the axons were shown. Bar = 2 μm. Quantification of the density of Synaptotagmin 1 and vGlut1 for experiments presented in p. n > 48 dendrites from three independent experiments per condition. *P < 0.05, ***P < 0.001. Student's t-test. K QI mice were transfected with plasmid encoding DsRed followed by immunodetection of synaptotagmin 1 (Syt1) and vGlut1 at DIV 18. Representative images of Syt1-positive puncta (green, Syt1), vGlut1-positive puncta (gray, vGlut1), and axon (red, DsRed) were shown. Imaris software processed Syt1 staining (green, Imaris Syt1) and Imaris software processed vGlut1 staining (white, Imaris vGlut1) on axons were shown. Bar = 2 μm. Quantification of the density of Synaptotagmin 1 and vGlut1 for experiments presented in p. n > 48 dendrites from three independent experiments per condition. *P < 0.05, ***P < 0.001. Student's t-test. K QI mice were transfected with plasmid encoding DsRed followed by immunodetection of synaptotagmin 1 (Syt1) and vGlut1 at DIV 18. Representative images of Syt1-positive puncta (green, Syt1), vGlut1-positive puncta (gray, vGlut1), and axon (red, DsRed) were shown. Imaris software processed Syt1 staining (green, Imaris Syt1) and Imaris software processed vGlut1 staining (white, Imaris vGlut1) on axons were shown. Bar = 2 μm. Quantification of the density of Synaptotagmin 1 and vGlut1 for experiments presented in p. n > 48 dendrites from three independent experiments per condition. *P < 0.05, ***P < 0.001. Student's t-test.
Axonal mitochondria dynamics are required for presynaptic release and short-term plasticity. However, molecular mechanisms of how mitochondrial dynamics modulates synapse formation and function are barely understood.

In this study, we demonstrate that PINK1-mediated Drp1\textsuperscript{S616} phosphorylation regulates mitochondrial dynamics to modulate the development and maturation of excitatory circuits and synaptic plasticity in the hippocampus and cortex. Three lines of evidence support this notion. First, PINK1 phosphorylates Drp1\textsuperscript{S616} to promote mitochondrial fission, increase mitochondrial density and mitochondria in dendritic protrusion and presynaptic bouton.

Second, cLTP induction elevates PINK1-mediated Drp1\textsuperscript{S616} phosphorylation and promotes dendritic mitochondrial fission. LTP induction and maintenance are suppressed in Pink1 KO or Drp1\textsuperscript{S616A} KI mice. Modulation of mitochondrial morphology likely allows its localization and energy supply to dendritic protrusions.

Finally, the contextual fear memory and spatial memory are impaired in Drp1\textsuperscript{S616A} KI mice, highlighting an essential role of Drp1\textsuperscript{S616} phosphorylation in learning and memory.

PINK1/parkin are well known to regulate mitophagy. PINK1 also regulates mitochondrial dynamics. We have recently shown that PINK1 directly phosphorylates Drp1\textsuperscript{S616}, therefore, controls...
mitochondrial fission. Consistently, overexpression of PINK1 increases mitochondrial fission in primary rat hippocampal neurons at DIV12. In Drosophila, PINK1/Parkin is shown to regulate mitochondrial morphological dynamics. This study suggests that PINK1 regulates mitochondrial fission via phosphorylating Drp1S616, resulting in modulation of matura-

tion of synaptic circuit and synaptic plasticity for memory formation and consolidation. One potential mechanism supported by this study is that PINK1/phosphor-Drp1S616-promoted dendritic mitochondrial fission to generate smaller...
mitochondria to trafficking to pre- and postsynaptic terminals to provide the required energy. The majority of the energy in neurons is consumed at synapses to support synaptic development and synaptic functions, such as synaptic transmission and plasticity. Mitochondria are recognized as the main powerhouse to fuel synaptic events by producing ATP. Dendritic mitochondria are shown to provide ATP to support synaptic protein translation during synaptic plasticity and to generate ROS to maintain the long-term phase of structural LTP. In contrast to this study, silencing PINK1 in primary mouse hippocampal neurons at DIV 7, the time point for active synaptogenesis prior to dendritic spines maturation, is hippocampal neurons at DIV 7, the time point for active contrast to this study, silencing PINK1 in primary mouse dependent or development-dependent manner. In this study, it is shown that PINK1 regulates mitochondrial dynamics in a context-dependent manner. Phosphorylation of Drp1 in multiple serines, including S585 and S616, prompted a rapid burst of Drp1-dependent-dendritic mitochondrial number, neuronal connectivity, and synaptic plasticity (illustrated in Fig. 8), providing a new avenue to study roles of mitochondria in establishment and refinement of the neuronal circuit and learning and memory.

**MATERIALS AND METHODS**

**Mice**

Pink1 KO mice were previously described. Methods for design of guide RNA, donor oligo, and production of Drp1S616A knockin mice were described. Briefly, the guide RNA target sequence (ATGCGAGAAATGGAATATCGAA) was selected according to the on-and-off-target scores from the CRISPR design web tool (http://www.benchling.com) and purchased from IDT (Coralville, Iowa, USA) in the two-part crRNA and tracrRNA format. To form a guide RNA duplex, we mixed crRNA and tracrRNA in 100 µl nuclease-free duplex buffer (IDT) at a final concentration of 1 mM each. The mix was heated at 95 °C for 5 min and allowed to cool on the benchtop to room temperature. To prepare the injection mixture, we incubated guide RNA duplex and Cas9 protein in 100 µl nuclease-free duplex buffer (IDT) at a final concentration of 1 mM each. The mix was heated at 95 °C for 5 min and allowed to cool on the benchtop to room temperature. To prepare the injection mixture, we incubated guide RNA duplex and Cas9 protein (IDT) at 37 °C for 10 min to form ribonucleoproteins and added the single-stranded DNA donor oligos (IDT) with S596A mutation (S596 of mouse Drp1 knockout mice) to the mixture. The mutant mice were generated by injection of the mixture into the cytoplasm of fertilized eggs of C57BL/6 genetic background, using a piezo-driven microinjection technique. Injected eggs were transferred into the oviductal ampulla of pseudopregnant CD-1 females on the same day. Pups were born and genotyped by PCR using the primers (5′-TGTGTTTCCATGCG-3′) and (5′-CCCAATCTCGTGCTCTCG-3′). The knockin allele was identified bySacII enzyme digestion and further confirmed by Sanger sequencing. Animals were housed in a controlled environment with a 12-h light/12-h dark cycle, with free access to water and a standard chow diet. Behavioral experiments were conducted between 10:00 a.m. and 5:00 p.m.

All animal procedures were carried out in accordance with either the Institutional Animal Care and Use Committee-approved protocol of Cincinnati Children’s Hospital or Medical Center or approved by the Ethics Review Committee for Animal Experimentation of Central South University.

**Primary neuronal cultures**

Cortical or hippocampal neuronal cultures were prepared from embryonic day 16.5 mice and plated on poly-d-lysine coated...
**PINK1-mediated Drp1<sup>S616</sup> phosphorylation.**

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**Figure a:**
- Merge, MitoGFP, DsRed, and Zoom panels show cellular staining patterns under different conditions.
- WT, Pink1 KO, Drp1<sup>S616</sup> KO, WT + Drp1<sup>S616</sup> KO, Pink1 KO + Drp1<sup>S616</sup> KO, and TEA treatments.

**Figure b:**
- Basal and TEA conditions compared.
- % of spine with mito.
- WT, Pink1 KO, Pink1 KO + Drp1<sup>S616</sup> KO, and WT + Drp1<sup>S616</sup> KO.

**Figure c:**
- Basal and TEA conditions compared.
- Mitochondrial length (μm).
- WT, Pink1 KO, Pink1 KO + Drp1<sup>S616</sup> KO, and WT + Drp1<sup>S616</sup> KO.

**Figure d:**
- LATENCY TIME vs. SESSION.
- WT and Drp<sup>S616</sup> KO conditions.

**Figure e:**
- 1h probe and 24h probe conditions.
- LATENCY TIME and CROSSING TIME.

**Figure f:**
- Habituation, 1 hour retention, and 24 hour retention.
- Freezing time (%).
- WT, Drp<sup>S616</sup> KO, Post-training, and Tested conditions.
coverslips as previously described.44 Briefly, hippocampi and cerebral cortices were isolated in cold HBSS and incubated with 0.025% trypsin at 37 °C for 20 min with gentle shaking every 5 min. After digestion, cells were dissociated with glass Pasteur pipettes ten times gently, filtered through 70 μm nylon cell strainer, and counted. Cells were plated at 4 × 10^5 cells/well for high density and 2 × 10^4 cells/well for low density in 12-well plates. Cultured cells were maintained in a neurobasal medium supplemented with 2% B27 and 2 mM glutamax at 37 °C and 5% CO₂.

Electrophysiology
Transverse brain slices (300 μm) were prepared from 6–8-week-old male mice as described previously.35 Slices were transferred to a holding chamber that contains artificial cerebrospinal fluid (124 mM NaCl, 3 mM KCl, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 1.2 mM MgCl₂, 10 mM glucose, 2 mM CaCl₂, pH 7.4, 305 mMosm, bubbled with 95% O₂/5% CO₂). The slices were allowed to recover at 31.5 °C for 30 min and then at room temperature for 1 h. Acute slices were transferred to a recording chamber continuously, which was perfused with oxygenated artificial cerebrospinal fluid (2 ml/min) at room temperature. For whole-cell patch-clamp recordings from the cortical layer 2/3 pyramidal cells, brain slices were visualized via input resistance-DIC by using an Axioskop 2FS equipped with Hamamatsu C2400-07E optics (Hamamatsu City, Japan). Basic electrophysiological properties were recorded when stable recordings were achieved with good access resistance (20 MΩ). The mEPSCs were recorded using an internal solution containing (in mM) 140 potassium gluconate, 10 HEPES, 0.2 EGTA, 0.3 mM Phosphocreatine sodium, 0.4% biocytin, 285-295 mOsm, 1 mM EGTA, 2 mM QX314-Cl, 4 mM Mg-ATP, 0.3 mM Na₃GTP, pH 7.25. The external solution contained 50 µM picrotoxin (Invitrogen, Carlsbad, USA).

LTP induction, hippocampal slices (300 μm) were prepared as described above. For field potential recordings, acute hippocampal slices were placed on a Med64-multielectrode array (Alpha MED Scientific, Osaka, Japan). Field excitatory postsynaptic potentials (fEPSPs) were elicited and recorded via planar electrodes of the Probe AL-MED-P515A by aligning the electrodes and the stratum radiatum region of hippocampal slices. An input-output curve analysis was performed at the beginning of each recording to determine the appropriate stimulation intensity. Test stimuli at 30–40% of maximal intensity were delivered at 0.05 Hz and a stable baseline of fEPSPs of 20 min was established before LTP induction. The amplitude of peak fEPSPs was measured. LTP was induced using a theta-burst protocol comprised of three trains delivered every 10 s, each train containing ten bursts at 5 Hz, each burst containing four pulses at 100 Hz. LTP was induced at 10 μA above test intensity to ensure robust LTP induction. Recordings lasted for an hour after induction. Recordings and analysis were performed using Med64 Mobius Software (Alpha MED Scientific).

Plasmids and transfection
Plasmid encoding C-terminal FLAG-tagged Pink1 was described previously.41 cDNA encoding Drp1 (Human isoform 1) was cloned into pcDNA3.1/myc-his(6) (Invitrogen, San Diego, USA). Constructs encoding Drp1S616A and Drp1S616D mutants were generated using a QuickChange site-directed Mutagenesis Kit from Stratagene (La Jolla, USA). For most experiments, cells were transfected using calcium phosphate or Lipofectamine2000 (Invitrogen, Carlsbad, USA).

Immunofluorescence and image analysis
Neurons were fixed with 4% (wt/vol) paraformaldehyde/4% (wt/vol) sucrose for 10 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature, blocked with 5% (vol/vol) BSA in PBS for 1 h at room temperature, and incubated with the primary antibodies in blocking solution (5% BSA in PBS) at 4 °C overnight. The secondary antibodies were diluted in blocking solution and incubated for 1 h at room temperature and mounted with Vectashield mounting medium (Vector, Burlingame, CA). The following antibodies were used in experiments: GFP (Aves: 1:400); PSD95 (Invitrogen; 1:200); Synaptophysin (Sigma; 1:200); Homer1 (Synaptic Systems; 1:500); Synapsin (Synaptic Systems; 1:500); and LABC (Bio-optical, Rochester, NY)

For immunostaining of brain slices, mice were anesthetized and perfused with ice-cold PBS followed by 4% (wt/vol)
Dissected mouse brain samples were post-fixed in 4% (wt/vol) paraformaldehyde for 24 h, then washed by PBS, and stored in 30% (wt/vol) sucrose solution at 4 °C. About 30 µm brain slices were coronally sectioned through a cryostat. For immunofluorescence, sections were permeabilized with 0.3% Triton X-100 in PBS for 30 min at room temperature, blocked with 5% (vol/vol) BSA in PBS for 1 h at room temperature. After blocking, sections were incubated with the primary antibody in blocking buffer at 4 °C overnight, followed by incubation with secondary antibodies for 1 h at room temperature and mounted with Vectashield mounting medium (Vector, Burlingame, CA). The following antibodies were used in tissue staining: PSD95 (Invitrogen; 1:1000); Synaptophysin (Sigma, St Louis, MO, USA; 1:1000); Homer1 (Synaptic Systems, Goettingen, Germany; 1:1000); Synapsin (Synaptic Systems, 1:1000).

To quantify the number of dendritic protrusions, Z-stacked images were converted to maximal projection images and analyzed using ImageJ (NIH, USA). The length of secondary dendrite was measured by using ImageJ and the number of each type of dendritic protrusions in the given dendrite was manually counted. Two to three dendrites from each cell were analyzed. Colocalized pre- and postsynaptic puncta, as well as axonal Synaptotagmin 1 and vGlut1 puncta were analyzed and 3D reconstructed by using the “spot” function of Imaris software (Bitplane Inc., Zurich, Switzerland).

Synaptotagmin 1 labeling in living cells
Exocytosed synaptic vesicles were labeled according to the previous report. Cortical neuronal cultures were incubated at 37 °C for 20 min with a monoclonal antibody against the luminal domain of Synaptotagmin 1 (Synaptic Systems, Goettingen, Germany; 1:200) diluted in culture medium. Neurons were washed with PBS to remove unbound antibody, fixed, and immunostaining with an antibody against vGlut1 (Synaptic Systems; 1:500). Images were acquired on the ZEISS LSM880 confocal system. Axonal Synaptotagmin 1 and vGlut1 puncta were 3D reconstructed and colocalization were calculated by using the “spot” function of Imaris software. (Bitplane Inc., Zurich, Switzerland).

ATP/ADP ratio analysis
To quantify the ratiometric signal of PercevalHR, the fluorescent intensity of 488 and 405 nm was analyzed by using ImageJ. The pseudo-color images reflecting the F488/F405 intensity were generated by using ImageJ.

Crude synaptosome isolation
Hippocampal and cortical tissues were homogenized in ice-cold crude synaptosome isolation buffer (1 mM MgCl2, 5 mM HEPES, 0.5 mM CaCl2, 0.32 M sucrose). Homogenized samples were centrifuged at 800×g for 10 min at 4 °C. Supernatants were transferred and centrifuged at 13,800×g for 10 min to get the pellet fraction that is a crude synaptosome.

Immunoblotting
Immunoblotting was performed as previously described. The following antibodies were used for this study: Phospho-Drp1 (Ser616) (D9A1) (CST, Danvers, MA, USA; 1:1000); Drp1 (D6C7) (CST; 1:1000); PSD95 (CST; 1:2000); Synaptophsyn (Abcam, Cambridge, UK; 1:2000); Tuj1 (Millipore, Burlington, MA, USA; 1:1000); Actin (Sigma; 1:5000); vGlut1 (Synaptic Systems; 1:500).

Chemical LTP induction
Chemical LTP was induced by perfusion with ACSF (pH 7.4, gassed with 95% O2/5% CO2, composed of in mM: 119 NaCl, 2.5 KCl, 26.2 NaHCO3, 1 NaH2PO4, 5 CaCl2, and 11 D-glucose) containing 25 mM TEA for 15 min. For immunoblotting analysis, neurons were treated with 25 mM TEA for 2.5, 5, and 15 min. After 15 min TEA treatment, TEA was washed followed by harvesting at 15 and 45 min. For immunofluorescence analysis, neurons were treated with TEA (25 mM, TEA) or solvent (Basal) for 15 min, followed by fixation.

Fig. 8  Schematic illustration of PINK1-mediated Drp1S616 phosphorylation to regulate synaptic transmission and plasticity via mitochondrial fission. Fused mitochondria (left top panel) become fished (left bottom panel) via a PINK1/phosphor-Drp1S616 mediated mechanism (left middle panel). Fused mitochondria are difficult to traffic to presynaptic terminals and dendrites (right upper panel) compared to fished mitochondria (right lower panel).
Golgi staining
Mice were perfused with ice-cold PBS. Brains were dissected and immediately transferred into the Bioenno Tech superGolgi kit (Bioenno Tech, Santa Ana, CA, USA) for the Golgi-Cox method of staining in accordance with the manufacturer’s instructions. Briefly, the samples were impregnated with the potassium dichromate and mercuric chloride solution at room temperature for 10 days. After impregnation, transfer them into Post-impregnation Buffer for 2 days post-impregnation. Brains were then serially sectioned at 150 μm using a vibrating microtome and sections were collected in Collection & Mounting Buffer. Sections were mounted on adhesive microscope slides. Slices were washed in 0.01 M PBS-T for 10 min and then placed in a diluted solution C (3:5 with H2O) for 30 min in a closed staining jar. After that, place slides in reagent D-prepared Post-staining Buffer for 20 min in a dark area, and then wash in 0.01 M PBS-T three times, for 10 min. Air-dry the slides out of direct light. Dehydrate sections in 100% ethanol for 10 min and repeat four times. Sections were cleared in xylene three times, 10 min per time, and mounted.

Transmission electron microscopy (TEM)
Mice were anesthetized and perfused with ice-cold PBS, followed by 2% (wt/vol) paraformaldehyde/2% (wt/vol) glutaraldehyde in 0.1 M sodium phosphate buffer, then immersed in osmium tetroxide (19150, Electron Microscopy Sciences, Hatfield, USA), dehydrated in ethanol (46139, Sigma-Aldrich, St. Louis, USA), and embedded in Epon (45345, Sigma-Aldrich, St. Louis, USA). After polymerization of Epon, blocks were sectioned to generate 70 nm thick sections using a diamond knife on a microtome (Leica, Wetzlar, Germany). The sections were stained with uranyl acetate (19481, TED PELLA, Redding, USA) and lead citrate (15326, Sigma-Aldrich, St. Louis, USA). Digital images were obtained on a Tecnai G2 Spirit by FEI equipped with an Eagle 4K HS digital camera.

Morris water maze (MWM)
The MWM was performed as previously described with minor modifications.50 The MWM was conducted in a circular pool (120 cm in diameter and 50 cm in height) with a featureless inner surface, filled with opaque nontoxic white dye. The water pool was placed in a dimly lit, soundproof test room surrounded by distinct extra maze cues. The pool was equally divided into four quadrants. A white platform was placed randomly in one of the quadrants and submerged 1 cm below the water surface to ensure that the platform was invisible. A pretraining day was dedicated for swim training (60 s) without the platform. This was conducted in the subsequent 5 consecutive days. During training days, 7-month-old Drp1S616A/KI male mice and WT control male littermates received two training sessions per day, with each session containing three trials. The trial location was changed semi-randomly between trials with the platform location fixed throughout the training process. After a mouse located the platform, it was permitted to stay on it for 10 s. A mouse was guided to the platform and placed on it for 10 s if it cannot locate the platform within 60 s. The mouse was taken to its home cage and allowed to dry up under a dim lamp after each trial. The time interval between each session was 30 min. Probe trials were conducted after the last training session (1 and 24 h after). During the probe trial, the platform was removed. Mice were allowed to swim for 60 s. All mice were placed at the same starting point, which was the farthest dropping location of the platform. During all trials, latency time, swim paths, crossing times, and percentage of time spent in each quadrant were recorded using a video camera-based Top-scan System (Clever Sys, Inc).

Fear conditioning
On day 1, 9-month-old mice were allowed to explore the test chamber (Clever Sys, Inc) for 2 min. On day 2, mice were placed in the same test chamber for 3 min, followed by exposed to the conditioned stimulus (CS; a 2800 Hz, lasting for 30 s and flash lasting for 3 s) co-terminated with the unconditioned stimulus (1.0 mA of continuous 1 s footshock). After two cycles, mice were detained for 3 min before being returned to the home cage.
To test for contextual fear memory, mice were placed in the training chamber and monitored for 3 min at 1 or 24 h after training. To test for cued fear memory, mice were placed in a novel chamber at 1 or 24 h post-training, and exposed to CS at 3 min after they were introduced into the chamber. Freezing was defined as the complete immobility of the animal, except for respiratory movement.

Statistics
Student’s t-test was used to determine the significance of the difference between the two groups. Statistical significance between each group with a control group was analyzed using one-way ANOVA followed by Dunnett’s multiple comparison test. All error bars indicate SEM. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.
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