Synaptotagmin-11 is a critical mediator of parkin-linked neurotoxicity and Parkinson’s disease-like pathology

Changhe Wang1,2, Xinjiang Kang1,3,4, Li Zhou1, Zuying Chai1, Qihui Wu1, Rong Huang1, Huadong Xu1, Meiqin Hu1, Xiaoxuan Sun1, Suhua Sun1, Jie Li1, Ruining Jiao1, Panli Zuo1, Lianghong Zheng1, Zhenyu Yue5 Zhuan Zhou1

Loss-of-function mutations in Parkin are the most common causes of autosomal recessive Parkinson’s disease (PD). Many putative substrates of parkin have been reported; their pathogenic roles, however, remain obscure due to poor characterization, particularly in vivo. Here, we show that synaptotagmin-11, encoded by a PD-risk gene SYT11, is a physiological substrate of parkin and plays critical roles in mediating parkin-linked neurotoxicity. Unilateral overexpression of full-length, but not C2B-truncated, synaptotagmin-11 in the substantia nigra pars compacta (SNpc) impairs ipsilateral striatal dopamine release, causes late-onset degeneration of dopaminergic neurons, and induces progressive contralateral motor abnormalities. Mechanistically, synaptotagmin-11 impairs vesicle pool replenishment and thus dopamine release by inhibiting endocytosis. Furthermore, parkin deficiency induces synaptotagmin-11 accumulation and PD-like neurotoxicity in mouse models, which is reversed by SYT11 knockdown in the SNpc or knockout of SYT11 restricted to dopaminergic neurons. Thus, PD-like neurotoxicity induced by parkin dysfunction requires synaptotagmin-11 accumulation in SNpc dopaminergic neurons.
Parkinson’s disease (PD) is a complex neurodegenerative movement disorder characterized by the selective vulnerability of dopaminergic neurons in the substantia nigra pars compacta (SNpc), and the impairment of dopamine (DA) release in the striatum. Numerous PD-risk genes and environmental factors have been identified, but their functional connectivity is poorly understood, thus hindering the elucidation of a common pathogenic pathway for PD. Parkin (encoded by PARK2) is an E3 ubiquitin ligase that regulates proteasome-dependent protein degradation and mitochondrial autophagy (mitophagy). It has been shown that mutations in parkin affect either its E3 ligase activity or its interactions with E2 enzymes, causing familial recessive and sporadic early-onset PD. Abnormal accumulation of parkin substrates may contribute to DA neuron degeneration in PD associated with parkin mutations. Although many putative substrates of parkin have been identified, due to a lack of unequivocal in vivo evidence, the involvement of these substrates in the pathogenesis of PD and the underlying mechanisms remain elusive.

A recent meta-analysis of PD populations based on five genome-wide association studies has identified 11 loci linked to a significant risk of PD, including synaptotagmin-11 (SYT11) as one of five novel PD-risk genes. A number of SYT proteins have been shown to be Ca<sup>2+</sup>-sensors for SNARE-mediated vesicle fusion during neurotransmitter release and hormone secretion. SYT1 and SYT4 have also been shown to function in endocytosis. We recently found that SYT11, a non-Ca<sup>2+</sup>-binding SYT, inhibits endocytosis and thus vesicle recycling in neurons. Its role in neurodegenerative disease, however, has never been reported. Interestingly, SYT11 can be ubiquitinated by parkin in vitro and accumulates in Lewy bodies in the sporadic human PD patient brain tissue. Here we show that SYT11 is a parkin substrate and mediates PD-like neurotoxicity and behavioral deficits.

**Results**

**SYT11 is a parkin substrate.** To determine whether SYT11 is a parkin substrate, we used co-immunoprecipitation and found an interaction between SYT11 and parkin in HEK293 cells over-expressing YFP-parkin and Myc-SYT11 (Fig. 1a, b). We next verified that parkin ubiquitinitates SYT11 in vitro. The analysis of anti-Myc immunoprecipitates from the lysates of HEK293 cells transfected with Myc-SYT11, YFP-parkin, and FLAG-ubiquitin (Flag-Ub) revealed that the ubiquitinitation of SYT11 was markedly increased by the overexpression of parkin (Fig. 1c). In addition, SYT11 expression was greatly reduced by parkin overexpression, while the pathogenic parkin mutations found in PD patients, such as R42P (disrupting the ubiquitin-like domain) and R275W (disrupting the RING1 domain), failed to mediate SYT11 degradation (Fig. 1d). Furthermore, the parkin-mediated decrease of SYT11 level was prevented by 12-h pretreatment with the proteasome inhibitor MG132 (Fig. 1d), confirming that parkin-mediated SYT11 degradation occurs through the proteasome pathway.

To further confirm that parkin also regulates endogenous SYT11 expression, we overexpressed YFP-parkin in hippocampal neurons and collected the transfected cells with a fluorescence-activated cell sorter. Western blot analysis revealed that the endogenous SYT11 expression in primary neurons was also decreased by parkin overexpression (Supplementary Fig. 1). Importantly, knockdown (KD) of parkin expression using a lentivirus carrying parkin shRNA (shParkin) induced the accumulation of SYT11 in the SNpc (Fig. 1e–j) and Supplementary Fig. 1a–c), while the scrambled shRNA served as a control (parkin<sub>Ipsi/Contra</sub>: 1.01 ± 0.24, P = 0.97; SYT11<sub>Ipsi/Contra</sub> = 1.02 ± 0.05, P = 0.73; n = 3), verifying the role of parkin in regulating SYT11 levels in vivo. Together with the previous in vitro data, our findings demonstrate that SYT11 is a parkin substrate, while parkin acts as the E3 ligase to directly regulate SYT11 protein levels through ubiquitin-dependent proteasome degradation.

**SYT11 accumulation induces behavioral deficits.** Since parkin deficiency and its pathogenic mutations induce the abnormal accumulation of SYT11 (Fig. 1), we next asked whether increasing SYT11 levels alone is sufficient to trigger PD-related toxicity in the mouse brain. SYT11-carrying lentivirus was delivered to the SNpc in the right hemisphere by stereotaxic injection (Fig. 2a). The results showed efficient infection of dopaminergic neurons in the SNpc, as evidenced by GFP expression in tyrosine hydroxylase (TH)-positive cells (Fig. 2b). Parkin expression remained largely unchanged in mice overexpressing SYT11 (Fig. 2d and Supplementary Fig. 2). Since SYT11 was unilaterally overexpressed in the SNpc region, we used methamphetamine (METH)-induced rotational asymmetry as a functional behavioral readout to assess the progression and severity of motor abnormalities induced by SYT11 overexpression. Mice injected with virus carrying GFP-only served as controls and they showed no defect of asymmetric rotation (Fig. 2e–h). Strikingly, unilateral overexpression (OE) of SYT11 induced a progressive increase in METH-induced contralateral rotation and a gradually shortened latency to rotation during 3–8 weeks after virus injection (Fig. 2e–h). Footprint gait analysis revealed that SYT11-OE mice also showed an unbalanced gait in SYT11-OE mice 1 month after virus injection (Fig. 2i–j). Mice with unilateral SYT11-OE showed greater variation of the contralateral stride length (Fig. 2i), which was similar on average to that of the ipsilateral side (contralateral, 7.93 ± 0.22; ipsilateral, 7.85 ± 0.18, P = 0.23), but with a larger standard deviation and range (Fig. 2j, k). Consistently, SYT11-OE mice also showed greater contralateral than ipsilateral front/hindpaw overlap (Fig. 2j). In contrast, both stride length and paw overlap were similar on both sides of the control virus-injected mice (Fig. 2m–p). Collectively, these findings indicate that SYT11 overexpression in this brain region is sufficient to induce locomotor deficits.

**SYT11 accumulation impairs DA release.** We next made amperometric recordings with electrochemical carbon fiber electrodes (CFEs) in striatal slices to determine whether SYT11-OE in the SNpc impairs DA release from nigrostriatal terminals. When a local electrical stimulus (Estim) was applied to the striatal slice, there was a transient increase in amperometric current (I<sub>amp</sub> with an amplitude of ~400 pA) with a subsequent decay to baseline, representing a transient increase in extracellular DA concentration (~2.4 μM, Fig. 3a and Supplementary Fig. 3). Strikingly, unilateral overexpression of SYT11 in the SNpc markedly decreased the DA release in the ipsilateral striatum when compared with that in the contralateral striatum of the same mice at 1 month after virus injection (Fig. 3a–c). In contrast, DA release in the ipsilateral striatum remained intact when control virus was used (Fig. 3d–f). In addition, SYT11-OE inhibited secretion in neuroendocrine adrenal chromaffin cells (Supplementary Fig. 4). Together, these results demonstrate that SYT11 overexpression in the SNpc in vivo is sufficient to cause a defect of DA release in the striatum.

**SYT11 accumulation leads to late onset loss of DA neurons.** To investigate whether SYT11 overexpression also leads to the loss of dopaminergic neurons in the SNpc, we performed stereological...
counting of TH-positive neurons in the Syt11-OE and contralateral sides of the SNpc. Interestingly, Syt11-OE failed to induce a clear loss of dopaminergic neurons in the SNpc at one month after injection (Supplementary Fig. 5), despite the robust motor impairment (Fig. 2) and DA release defects (Fig. 3a–f) at this stage. However, we observed ~35% loss of DA neurons in the SNpc with Syt11-OE at 3 months after injection, while no significant loss of TH-positive neurons was found when control virus was used (Fig. 3g, h). We also found positive terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining in the Syt11-OE, but not in the contralateral SNpc (Fig. 3g), suggesting that these DA neurons degenerate through

![Diagram](image)

**Fig. 1** Effect of Parkin dysfunction on Syt11 accumulation. a, b Co-immunoprecipitation of Syt11 with parkin. YFP-parkin (or GFP) and Myc-Syt11 was co-expressed in HEK293 cells, and the lysates were immunoprecipitated (IP) with anti-GFP or anti-Myc antibodies, followed by immunoblotting (IB) with antibodies as indicated. Whole-cell lysates were also immunoblotted with the indicated antibodies as controls. c Parkin catalyzes the ubiquitination of Syt11. Ubiquitination of Syt11 in HEK293 cells expressing combinations of Myc-Syt11, YFP-parkin, and Flag-ubiquitin (Flag-Ub) as indicated. Cell lysates were immunoprecipitated with anti-Myc, followed by immunoblotting with anti-Flag antibody. d Parkin mediates proteasome-dependent degradation of Syt11. Immunoblotting for Syt11 in HEK293 cells expressing YFP-parkin or parkin mutants (R42P disrupting the ubiquitin-like domain, or R275W disrupting the RING1 domain of parkin), with or without 12 h treatment with MG132 (10 μM). Data were collected from three independent experiments (P = 0.041 for ANOVA, P < 0.001 for further LSD test between Myc-Syt11/YFP-parkin and Myc-Syt11). e Cartoon of virus injection for unilateral parkin knockdown (KD) and parkin/syt11 double-KD in the SNpc. f Representative western blots showing the expression of parkin, Syt11, and TH in the bilateral SNpc from parkin KD and parkin/syt11 double-KD mice 1 month after virus injection as in e. g–j Statistics of parkin and Syt11 expression in the SNpc from mice as indicated (P = 0.000, 0.040, 0.047, and 0.022; number in parentheses represents number of mice/biological repeats). Data are shown as mean ± s.e.m. One-way ANOVA for d and paired Student’s t-test for g–j, *P < 0.05, ***P < 0.001.
Fig. 2 Unilateral Syt11 overexpression in the SNpc induces motor abnormalities. a Schematic of unilateral virus injection in the SNpc and time course of experiments. b Representative micrograph of TH staining in an SNpc-containing slice showing that GFP-carrying lentivirus was precisely injected into the SNpc (ipsi) and efficiently infected the dopaminergic neurons. Scale bars, 500 μm. c-f Representative western blots and statistics showing the expression of Syt11 in the bilateral SNpc from mice overexpressing (OE) Syt11 unilaterally and controls 1 month after virus injection (Control, P = 0.691; Syt11-OE, P = 0.022). g, h Methamphetamine (METH) induces progressive asymmetric rotation (rotation latency and rotations in 90 min) of mice with unilateral OE of Syt11 in the SNpc, compared with control virus (latency, P = 0.022, 0.000, 0.000, and 0.000 for 3, 4, 6, and 8 weeks; rotation, P = 0.020, 0.007, 0.003, and 0.000 for 3, 4, 6, and 8 weeks). i-l Footprint data showing the defect in contralateral motor stability of mice with unilateral Syt11-OE in the SNpc. Stride length (j-k, SD, P = 0.003; range, P = 0.004) and front/hind footprint overlap (l, P < 0.001, n = 88 Contra and 84 Ipsi steps from six mice) were quantified, and the dashed red lines in l represent the 5 and 95% percentiles of stride length. m-p Footprint data showing the normal motor stability of mice unilaterally expressing control virus in the SNpc (SD, P = 0.946; range, P = 0.347; foot overlap, P = 0.857, n = 50 Contra and 50 Ipsi steps from six mice). Contra, contralateral; Ips, ipsilateral. Data are shown as mean ± s.e.m. for e-h, j-k, n-o. Paired Student’s t-test for e, f, j, k, n-o, unpaired Student’s t-test for g, h, Mann-Whitney test for l, p, box and whisker plots show medians (central line in the box), ranges between 25th and 75th percentiles (box), and minimum-maximum ranges (whiskers). *P < 0.05, **P < 0.01, ***P < 0.001.
apoptosis. Consistently, the reduced fluorescence intensity of TH staining also revealed a substantial reduction of TH-positive neurites in the ipsilateral striatum (Fig. 3i, j). Thus, our data indicate that Syt11 overexpression also induces the progressive loss of dopaminergic neurons, which is preceded by depression of DA release.

**Syt11 inhibits endocytosis and vesicle replenishment.** An independent study from our lab has shown that syt11 inhibits dynamin-dependent endocytosis by limiting the membrane invagination in dorsal root ganglion and hippocampal neurons, so we next determined whether the impaired DA transmission was also due to the inhibitory role of Syt11 in endocytosis in dopaminergic neurons. Alexa Fluor-conjugated
transferrin uptake was measured to evaluate clathrin-mediated endocytosis (CME) in Syt11-overexpressing dopaminergic neurons in the SNpc of TH-driven GFP transgenic mice. We found that, compared with the contralateral side, transferrin uptake was markedly suppressed in TH-positive neurons in the Syt11-OE SNpc (Fig. 4a and Supplementary Fig. 6), indicating that CME was slowed due to Syt11-OE. To further verify the inhibitory role of Syt11 in neuronal endocytosis, we used total internal reflection fluorescence (TIRF) imaging of clathrin-ΔSRed in the somata of hippocampal neurons with or without Syt11 overexpression. Strikingly, most of the assembled clathrin clusters were clamped at the plasma membrane, and thus the CME events were markedly reduced in Syt11-OE neurons (Fig. 4b and Supplementary Fig. 7). Furthermore, we used live imaging of the synaptophysin-phluorin reporter, an assay for stimulated synaptic endocytosis/exocytosis (Fig. 4c), to monitor the function of Syt11 in endocytosis and vesicle recycling. Consistently, Syt11-OE neurons showed a robust reduction in endocytic rate with a significantly longer time constant, confirming the inhibitory role of Syt11 in synaptic endocytosis and vesicle recycling of hippocampal neurons (Fig. 4d–f). These findings indicate an inhibitory action of Syt11 on endocytosis in DA neurons.

DA release in response to paired-pulse stimulation was used to further assess the role of Syt11 in vesicle recycling in DA neurons. As expected, we found a reduced paired-pulse ratio of DA release in ipsilateral striatal slices overexpressing Syt11 (Fig. 5a and Supplementary Fig. 8), indicating that the vesicle replenishment was also inhibited. Burst stimulation using a train of 10 pulses at 20 Hz also revealed a reduced releasable vesicle pool in the striatal DA terminals on the Syt11-OE side (Fig. 5b and Supplementary Fig. 9a–c). In addition, we re-examined the vesicle recycling rate after depleting the releasable vesicle pools and confirmed the slowed vesicle replenishment by Syt11 (Fig. 5c and Supplementary Fig. 9). Furthermore, we crossed homozygous floxed Syt11-null mice with heterozygous DA transporter-driven Cre-knockin mice (DAT-Cre) to produce DA neuron-restricted Syt11 conditional knockout (Syt11-cKO) mice (Fig. 5d). As expected, immunostaining showed a nearly complete loss of Syt11 in TH-positive neurons in the ventral midbrain, while Syt11 expression in TH-negative neurons remained intact (Supplementary Fig. 10a, b), indicating a specific knockout of Syt11 in dopaminergic neurons. Consistently, western blots showed a dramatic reduction of Syt11 expression in the whole ventral midbrain (Supplementary Fig. 10c–e). Strikingly, amperometric recordings demonstrated increased DA release (Fig. 5e), accelerated vesicle replenishment (Fig. 5f), and enlarged releasable vesicle pools (Fig. 5g, h) in the striatum of Syt11-cKO mice, confirming the inhibitory role of Syt11 in vesicle recycling in DA neurons.

Structure–function analysis revealed that the C2B domain of Syt11 is critical for its inhibitory role in endocytosis. We next investigated whether this domain is also essential for Syt11 to mediate PD-related neurotoxicity (Fig. 6a). Strikingly, unilateral overexpression of the C2B-truncated form of Syt11 in the SNpc (Fig. 6b–d) failed to impair the ipsilateral striatal DA release (Fig. 6e, f) and the contralateral motor stability (Fig. 6g–k), indicating the critical role of the C2B domain in the Syt11-mediated pathogenesis of PD. Collectively, these findings suggest that Syt11 plays an important role in DA transmission by regulating endocytosis and the vesicle-recycling process, while the upregulation/accumulation of Syt11 causes the reduction of releasable vesicle pools, impairment of DA release in the striatum, and thus the progression of PD.

**Syt11 is essential for the parkin-linked neurotoxicity.** Considering the possible developmental compensation in parkin-KO mice, to test the idea that Syt11 accumulation mediates the pathogenic role of parkin in PD, we knocked down both Syt11 and Parkin expression. We co-injected lentivirus expressing shParkin and lentivirus expressing Syt11 shRNA (shSyt11) or shRNA control (1:1 ratio) unilaterally into the SNpc (Fig. 7a). The combination of the two GFP- and RFP-expressing scrambled shRNA control lentiviruses showed high co-infection efficiency (>80%) in the midbrain in vivo (Supplementary Fig. 11). Immunoblotting showed the abnormal accumulation of Syt11 in the shParkin (plus control virus)-injected SNpc (Fig. 1c–h and Supplementary Fig. 12a–c). Co-injection of virus carrying shParkin and shSyt11 abolished the accumulated Syt11 expression due to parkin KD only (Fig. 1e–j). Consistent with the impaired DA release in parkin-KO mice, amperometric recording showed the decreased nigrostriatal DA release caused by parkin KD in the SNpc at 1 month after injection (Fig. 7b–d and Supplementary Fig. 12d, e). Interestingly, additional Syt11 KD completely reversed the parkin KD-induced impairment of striatal DA release (Fig. 7e–g). These results demonstrated the critical role of Syt11 accumulation in the reduced DA release caused by parkin deficiency. Furthermore, TH staining analysis showed that parkin KD induced a remarkable loss of DA neurons in the SNpc, which was reversed by parkin/Syt11 double-KD at 3 months after virus injection (Supplementary Fig. 13). METH induced progressive asymmetric rotation in mice with unilateral KD of parkin in the SNpc during 8 weeks after virus injection, and this abnormal motor activity was attenuated in mice with double-KD of parkin and Syt11 (Fig. 7h, i and Supplementary Fig. 12f). In addition, the impaired contralateral motor stability also occurred in mice with unilateral parkin KD in the SNpc (Fig. 7j–l and Supplementary Fig. 12g–i), but was prevented by Syt11/parkin double-KD (Fig. 7m–o). These results establish that Syt11 accumulation is critical for parkin to mediate PD-related neurotoxicity.

Since the PD-related neurotoxicity could be due to the direct defects of the SNpc DA neurons or indirect effects of other cells in this region, we sought to determine whether the Syt11 accumulation in DA neuron is the major cause of the neuronal toxicity in PD. We unilaterally overexpressed Syt11 in SNpc DA neurons and this abnormal motor activity was attenuated in mice with double-KD of parkin and Syt11 (Fig. 7h, i and Supplementary Fig. 12f). In addition, the impaired contralateral motor stability also occurred in mice with unilateral parkin KD in the SNpc (Fig. 7j–l and Supplementary Fig. 12g–i), but was prevented by Syt11/parkin double-KD (Fig. 7m–o). These results establish that Syt11 accumulation is critical for parkin to mediate PD-related neurotoxicity.
neurons by injecting a FLExloxP-based Flag-Syt11-overexpressing AAV9 virus into the right SNpc of DAT-Cre mice. Virus expressing FLExloxP-based GFP only served as a control (loxp control, Fig. 8a–f). TH staining showed specific overexpression of Syt11 (or GFP-only) in DA neurons in the SNpc on the injected side (Fig. 8a). Western blots confirmed the expression of Flag-Syt11 3 weeks after virus injection, which did not affect the expression of endogenous Syt11 in this region (Fig. 8b). Interestingly, the conditional overexpression of Syt11 in SNpc DA neurons induced a dramatic decrease in ipsilateral striatal DA release (Fig. 8c) and an imbalance of contralateral motor stability (Fig. 8d). Importantly, we also found that unilateral parkin KD in the SNpc (Fig. 8g) failed to impair the ipsilateral striatal DA release (Fig. 8h) and contralateral motor stability (Fig. 8i–m) in Syt11-cKO mice, demonstrating that Syt11 expression and accumulation in SNpc DA neurons is required for the pathogenesis caused by parkin deficiency.

**Discussion**

Although loss-of-function mutations in PARK2 are the most common causes of autosomal recessive PD, the mechanisms underlying parkin-associated PD have puzzled the entire field for more than two decades. A major obstacle for mechanistic studies is that parkin-KO mice show minimal defects in striatal DA release and no loss of DA neurons in the SNpc, most probably due to compensation for parkin deficiency during development, because parkin KD (Fig. 7 and Supplementary Figs. 12 and 13) or conditional parkin-KO in the SNpc produced similar PD neurotoxicity in adult mice. Here we produced a new adult mouse model for parkin-linked PD that is distinct from the traditional KO approaches and may represent the real conditions of PD with parkin mutations, which deserves a more thorough confirmation in future.

Many putative substrates of parkin have been reported, but their pathogenic roles remain to be established due to scarce evidence in vivo. Our findings, for the first time, reveal that Syt11, a PD-risk factor based on meta-analysis, is a physiopathological parkin substrate that mediates parkin-linked PD-like pathogenesis. Parkin dysfunction leads to the accumulation of Syt11, which inhibits endocytosis, vesicle replenishment, DA release from DA neurons, and finally initiates the pathogenesis of PD (Fig. 9). In addition to decreased DA transmission, the impaired endocytosis and vesicle recycling may also lead to a more general disturbance of the homeostasis of synaptic structure and membrane proteins, thus whether the impaired DA release can fully explain the Syt11-mediated pathogenesis needs further investigation (Fig. 9). Meanwhile, other parkin substrates may also cooperate with Syt11 to mediate the pathogenesis of parkin-linked PD and thus contribute to parkin-linked neurotoxicity. Collectively, the present work not only validates Syt11 accumulation as a PD-risk factor but also uncovers a novel pathogenic pathway/mecanism for parkin-associated PD.

Many PD-risk genes and environmental factors have been identified, but it has been challenging to identify a central pathogenic pathway for PD. Our study reveals functional
connectivity of the two PD-risk genes (parkin and SYT11) involving dysfunctional dopamine transmission, which is likely a common pathogenic pathway shared by other PD-risk genes (i.e. synaptojanin 1, α-synuclein, TMEM230, and LRRK2). Importantly, the progressive loss of DA neurons in the SNpc is markedly preceded by depression of DA transmission in Syt11-OE mice, indicating that the impaired endocytosis and vesicle recycling should be an early-stage event during PD pathogenesis. Our findings provided the first direct in vivo evidence that Syt11 mediates parkin-linked PD-like symptoms in mice and that the impaired vesicle recycling probably serves as a general early-stage pathogenic pathway for genetic causes of PD.

Methods

Plasmids. Myc-Syt11 was generated by PCR amplification of full-length rat synaptotagmin-11 (Syt11, AF000423) from pCMV5-Sty11 and then ligated into pCMV5-Myc (Clontech). YFP-parkin, pcDH-ParkinR42P, and pcDH-ParkinR275W were kindly provided by Thomas L. Schwarz (Children’s Hospital Boston and Harvard Medical School) with the permission of Richard J. Youle (National Institutes of Health) and Matthew LaVoie (Brigham and Women’s Hospital and Harvard Medical School). Flag-Ub was kindly provided by Ruiping Xiao (Peking University). Synaptophysin-pHluorin was kindly gifted by Yongling Zhu (The Salk Institute for Biological Studies). For in vivo experiments, we used a lentiviral system, pHUGW, kindly provided by Chen Zhang (Peking University) with the permission of Thomas C. Sudhof (Stanford University), by substituting the EGFP coding sequence with that of Syt11-IRES2-EGFP or Syt11 (for Tf uptake). A short-hairpin sequence targeting Syt11 (5'-GGCTGAGATCACAAATATACG-3') was cloned into pHUGW itself served as control. A short-hairpin sequence targeting Syt11-LoxP (5'-GGCTGAGATCACAAATATACG-3') was cloned into pHUGW itself served as control. A short-hairpin sequence targeting Syt11-LoxP (5'-GGCTGAGATCACAAATATACG-3') was cloned into pHUGW itself served as control.
Overexpression of C2B-truncated Syt11 in the SNpc fails to mediate deficits in DA release and motor behavior. a Schematic of unilateral overexpression of C2B-truncated Syt11 (ΔC2B) in the SNpc with an AAV9-expressing system. b Representative western blots showing the expression of parkin and Syt11 in the bilateral SNpc from mice with unilateral overexpression of ΔC2B as in a. Western blots were performed 1 month after virus injection. c, d Statistics showing the intact DA release from ΔC2B-overexpressing dopaminergic terminals in the striatum (P = 0.332, n = 9 pairs in slices from five mice). 

Footprint data showing that unilateral overexpression of ΔC2B in the SNpc fails to induce a motor defect in the contralateral limbs (SD, P = 0.900; range, P = 0.516; overlap, P = 0.917, n = 90 Contra and 85 Ipsi steps from 7 mice). Dashed red lines show the 5 and 95th percentiles of stride length in h. Data are shown as mean ± S.E.M. for c, d, f, g, i, j. *: one-way ANOVA for c, g. Mann–Whitney test for k, box and whisker plots show medians (central line in the box), ranges between 25th and 75th percentiles (box) and minimum-maximum ranges (whiskers). *P < 0.05, **P < 0.01, ***P < 0.001

Syt11 expression (shSyt11), and that targeting 5′-CCATCAAGAAGACCCACCA-3′ or 5′-CGATTCTGACACCAGCATTT-3′ was cloned into pMAGie 4.1 for the KD of parkin (shParkin and parkin sh-2). Lentiviruses carrying shSyt11, shParkin, or shRNA control were produced by Shanghai Sunbio Biomedical Technology Co., Ltd (China). The C2B domain (208–430)–truncated form of Syt11ΔC2B was sub-cloned into the Xhol–MluI site of the vector pHBAAV-CAG-ZsGreen. The 3XFlag-tagged Syt11 fragment was sub-cloned into the Kpnl–MluI site of the vector pHBAAV-CAG-DOI-ZsGreen to generate plasmids overexpressing Cre-inducible Syt11. The AAV9 viral systems were from Hanbio Biotechnology Co., Ltd (China). All constructs were verified by DNA sequencing.

Antibodies. The following primary antibodies were used: anti-β-actin (A5316; Sigma, 1:5000 dilution), anti-Syt11 (270.003; Synaptic System, 1:1000 dilution), anti-c-Myc (sc-789; Santa Cruz, 1:200 dilution, 1 μg ml⁻¹ for IP), anti-c-Myc (M 4439; Sigma, 1 μg ml⁻¹ for IP), anti-GFP (A1122; Invitrogen, 1:1000 dilution), anti-parkin (sc-32,282; Santa Cruz, 1:400 dilution), anti-Flag (F1804; Sigma, 1:1000 dilution), and anti-tyrosine hydroxylase (AB152; Millipore, 1:1000 dilution). IRDye 680CW goat anti-mouse IgG (LI-COR-680; LI-COR Biosciences, 1:5000 dilution) and IRDye 800CW goat anti-rabbit IgG (LI-COR-800; LI-COR Biosciences, 1:5000 dilution) and IRDye 800CW goat anti-rabbit IgG (LI-COR-32,211; LI-COR Biosciences, 1:5000 dilution) were used for immunoblotting. Alexa Fluor® 488 goat anti-rabbit IgG (H+L) (A11034, 1:5000 dilution), Alexa Fluor® 594 goat anti-rabbit IgG (H+L) (A11037, 1:5000 dilution), and Alexa Fluor® 633 goat anti-rabbit IgG (H+L) (A-21,071, 1:5000 dilution) were used for immunostaining.

Animals. The foxed Syt11-null mice were from The Jackson laboratory (strain B6.129-Syt11tm1mlsd/J). TH-GFP and DAT-Cre transgenic mice were kindly provided by Dr. Minmin Luo (National Institute of Biological Sciences, China). Mice were housed in the animal facility with a maximum of five mice per cage under a 12-h light/dark cycle at 22 ± 3 °C. Food and water were available ad libitum. The use and care of animals was approved and directed by the Animal Care and Use Committee of Peking University and the Association for Accreditation and Accreditation of Laboratory Animal Care.

Cell culture and transfection. Human embryonic kidney 293A (HEK293A) and HEK293T cells were originally sourced from the ATCC and regularly checked for mycoplasma contamination. They were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified incubator (5% CO₂). HEK293T cells were used for lentivirus production. For HEK293A cells, plasmids were transfected using VigoFect (Vigorous Biotechnology Beijing Co., China) when cells reached ~75% confluence following the manufacturer’s instructions. Immunoblotting and co-immunoprecipitation were performed 36 h after transfection.

Hippocampal neurons were prepared and cultured as described previously. Briefly, hippocampi were dissected from postnatal day 0–1 Wistar rats and treated with 0.25% trypsin at 37 °C for ~12 min. Cells were plated on polyethyleneimine-coated glass coverslips and maintained in DMEM (Gibco) supplemented with 10% FBS for 3 h, which was then replaced by Neurobasal (Gibco) supplemented with 2% B27, 0.5 mM l-glutamine, and 5 μM cytosine arabinoside. Cultures at 5 days
in vitro (DIV 5) were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. TIRF and confocal imaging were performed at DIV 14.

Rat adrenal chromaffin cells (RACCs) were cultured as previously described with slight modifications. Adult Wistar rats (female, ~250 g) were used and RACCs were isolated and digested in ice-cold D-Hanks containing collagenase type 1A (1 mg ml$^{-1}$), DNase (10 mg ml$^{-1}$), and hyaluronidase (2 mg ml$^{-1}$) at 37 °C for 40 min. Cells were dissociated, collected, and transfected with the NeonTM 10 μl transfection system MPK1096 (Invitrogen). Cells were then plated on poly-L-lysine-coated coverslips and maintained in DMEM (Gibco) supplemented with 10% FBS (Gibco) and cysteine (6 mg l$^{-1}$) at 37 °C in a humidified incubator (5% CO$_2$).
Lentivirus production and in vivo infection. Syt11-carrying and pFUGW control lentiviruses were produced as previously described. The lentiviral-expression vector and three helper plasmids (pRSV-REV, pMD1-g-pRE, and vesicular stomatitis virus G protein-expressing plasmid) were co-transfected into HEK293T cells at 4, 2, 2, and 2 μg of DNA per 25 cm² culture area, using polyethyleneimine “Max” (Mw 40,000, Cat. 24,765-2; Polyscience) following the instructions of the manufacturer.

Forty-eight hours after transfection, the culture medium was collected and clarified by centrifugation at 3000 g for 5 min. The supernatant was further centrifuged at 50,000 g for 120 min at 4 °C. The pellet was re-suspended in ice-cold phosphate balanced saline (PBS, 1%v/v volume of culture medium).

Stereotactic viral injections were carried out as described previously with small modifications. The mice were anesthetized with urethane (1.5 g kg⁻¹, i.p.) and body temperature was maintained at 37 °C using a heating pad (KEL-2000, Nanjing, China). Mice were fixed in a stereotactic frame (Narishige, Japan) and leveled using the bregma and lambda landmarks. Craniotomies were performed to cause minimal damage to cortical tissue. A total of 2 μl viral suspension (titer: 10⁻⁸ to 10⁻¹⁰ per ml) was injected into the SNpc (3 mm posterior from bregma, 1.25 mm lateral, 4 mm ventral from the dura) at a rate of 100 nl min⁻¹ using a 10 μl syringe with a blunt 32G needle. The needle was allowed to remain in place for another 20 min after injection, and then slowly withdrawn. Afterwards, the scalp was sutured and animals were placed on a 37 °C plate for recovery.

Behavioral assays. All behavior experiments were performed in the afternoon. METH-induced asymmetric rotation tests were carried out 3, 4, 6, and 8 weeks after virus injection. The mice were placed in a quiet place for 30 min to habituate to the environment before intraperitoneal METH injection (8 mg kg⁻¹). An unbiased observer counted the number of rotations for 90 min after injection. Rotation latency was recorded as the time from the METH injection to the appearance of the first asymmetric rotation and scored as 90 min if there was no rotation during the test.

Footprint gait was analyzed as previously described with slight modifications. The hindpaws and forepaws were coated with black and red non-toxic paint. The mice were trained to walk along a 100-cm-long and 20-cm-wide open-top runway (with 10-cm high walls) with three runs per day for three consecutive days. A fresh sheet of white paper was placed on the floor of the runway for each run. The footprint patterns were assessed quantitatively by stride length and front/hind footprint overlap.

Amperometric DA recordings in striatal slices. Amperometric recordings in dorsal striatum slices were made using CFEs as described previously. Mice were anesthetized with urethane (1.5 g kg⁻¹, i.p.) and transcardially perfused with a 50 ml ice-cold artificial cerebrospinal fluid (sectioning aCSF) containing (in mM): 110 NaCl, 2.5 KCl, 7.5 MgCl₂, 1.3 NaH₂PO₄, 25 NaCO₃, 25 glucose (saturated with 95 O₂ and 5% CO₂), then the brain was rapidly removed and cut on a vibratome into 500-μm-thick slices on a vibratome (Leica VT 1000s; Nussloch, Germany). Slices containing the striatum were collected at +0.0 to +1.2 mm from the bregma. Slices were allowed to recover for 30 min in “recording aCSF” (in mM): 125 NaCl, 2.5 KCl, 2 CaCl₂, 1.3 MgCl₂, 1.3 NaH₂PO₄, 25 NaCO₃, 10 glucose (saturated with 95 O₂ and 5% CO₂) at 37 °C, and then kept at room temperature for recording. CFEs 7 μm in diameter with a ~200-μm sensor tip were anesthetized with urethane (1.5 g kg⁻¹, i.p.) and post-fixed overnight in 4% paraformaldehyde in PBS at 4 °C. The slices were subsequently fixed in a cryo-solution of 4% formaldehyde and mounted on slides with 50% glycerol. A ×42 oil lens on a Zeiss 710 inverted confocal microscope was used to scan z-stack of 1-μm optical sections. Identical settings were applied to all samples in each experiment. The average fluorescence intensity of the Ti signal in GFP-positive cells body in the SNpc was calculated with ImageJ software.

TIRF imaging. Hippocampal neurons transfected with plasmid expressing Chlathrin-DsRed with or without the co-transfection of plasmid expressing Syt11 were cultured for 12-18 h. TIRF imaging was performed on an inverted microscope with a ×100 TIRF objective lens (Olympus IX-81; numerical aperture 1.45) as described previously. Images were captured by an Andor EMCCD using Andor iQ software with an exposure time of ~100 ms. The temperature of the cell underneath the imaging area was kept at ~35 °C by a laboratory-made heater attached on the objective lens. Standard or 70 mM KCl-containing external solution (in mM, 85 NaCl, 70 KCl, 2.5 CaCl₂, 1 MgCl₂, 10 H-HEPES, 10 mM NaClO₃, and 10 mM NaClO₃, pH 7.4) was applied using a gravity-fed perfusion system (MPS-2, Yibo Inc., Wuhan, China). For the analysis of single endocytic events, each event was selected and marked with 2.8-μm diameter circular area. Fluorescence intensity values were calculated and analyzed using ImageJ software. The whole-cell endocytosis was calculated based on the fluorescence changes of chlathrin-DsRed on the entire cell surface.

Confocal live imaging. Hippocampal neurons were continuously perfused with standards bath solution containing: 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM H-HEPES, 10 mM NaClO₃, 50 mM D-AP5, pH 7.4. Neurons were stimulated with 100 pulses delivered at 20 Hz (100 mA, 1-ms duration) and the output frequency was increased by 10 Hz per minute. The ratiometric reporter, RFP-GFP, was used to monitor neuronal activity. Images were acquired every 2 s. Merged images were converted to fluorescence intensity images through an intensity threshold of 100 arbitrary units.

**Fig. 7** Syt11 knockdown rescues parkin-induced motor defects in DA release and motor behavior. a Schematic of unilateral parkin knockdown (KD) and parkin/syt11 double-KD in the SNpc and time course experiments. b Unilateral KD of parkin in the SNpc reduces DA release in the ipsilateral striatum. **c** Incubation with 3,4-DHT for 18 h reverses the reduction of DA release by parkin KD in the ipsilateral striatum (amplitude, P = 0.001; charge, P = 0.008; n = 9 pairs in slices from five mice). e-g Syt11 KD reverses the reduction of DA release by parkin KD in the ipsilateral striatum (amplitude, P = 0.829; charge, P = 0.916; n = 10 pairs in slices from six mice). h, i METH-induced asymmetric rotation (latency and rotations in 90 min) in mice with unilateral parkin KD or parkin/Syt11 double-KD in the SNpc (latency, P < 0.001 for all time points; rotation, P = 0.001, 0.005, 0.000, and 0.002 for 3, 4, 6, and 8 weeks). j-o Footprint data showing that Syt11 KD reverses the parkin KD-induced motor defect in the contralateral limbs; dashed red lines show the 5 and 95% percentiles of stride length in j and m. n = 77 Contra and 77 Ipsi steps from seven parkin KD mice (P < 0.001); n = 87 Contra and 90 Ipsi steps from six DKO mice (SD, P = 0.420; Overlap, P = 0.797). Data are shown as mean ± s.e.m for c-g, k, n. Contra, contralateral; Ipsl, ipsilateral. Paired Student’s t-test for c, d, f, g, k, n, unpaired Student’s t-test for h, i, Mann–Whitney t-test for l, o. box and whisker plots show medians (central line in the box), ranges between 25th and 75th percentiles (box) and minimum–maximum ranges (whiskers). *P < 0.05, **P < 0.01, ***P < 0.001.
**Fig. 8** Expression of Syt11 in dopaminergic neurons is critical for parkin to mediate DA release and motor deficits. a Representative micrograph of TH staining in an SNpc-containing slice from DAT-Cre mice unilaterally injected with Cre-inducible Flag-Syt11 (or GFP control) overexpressing AAV9 virus. Scale bar, 500 μm. b Representative western blots and statistics showing the expression of Syt11 and Flag-Syt11 (F-Syt11, OE) in the bilateral SNpc from Syt11 cOE mice 1 month after virus injection. c METH-induced asymmetric rotation (in 90 min) of control (Ctrl) and Syt11 cOE mice. d Footprint gait analysis of Syt11 cOE and loxp control mice; dashed red lines show the 5 and 95% percentiles of stride length. n represents steps from seven control (e) or six Syt11 cOE mice (f). g Representative western blots and statistics showing the efficiency of parkin KD in the SNpc of Syt11-cKO mice. h Representative amperometric currents and statistics showing the unchanged DA release in the striatum of Syt11-cKO mice with unilateral parkin KD in the SNpc (n = 13 pairs in slices from five mice). i METH-induced asymmetric rotation of control mice with unilateral parkin KD or parkin/Syt11 double-KD in the SNpc, and that of Syt11-cKO mice with unilateral parkin KD. j-m Footprint data showing that unilateral parkin KD in the SNpc fails to induce a motor defect in the contralateral limbs of Syt11-cKO mice (n = 75 Contra and 77 Ipsi steps from five mice). Dashed red lines show the 5 and 95% percentiles of stride length in j. Data are shown as mean ± s.e.m for b, c, g, i. Paired Student’s t-test for b lower left, c, g, h, k, l, one-way ANOVA for b lower right, f; Mann-Whitney test for d-f, m; box and whisker plots show medians (central line in the box), ranges between 25th and 75th percentiles (box) and minimum-maximum ranges (whiskers). *P < 0.05, ***P < 0.001.
Fig. 9 Schematic model showing the role of Syt11 in parkin-associated PD. The E3 ligase parkin mediates the ubiquitination and proteasome-dependent degradation of Syt11, whereas parkin dysfunction induced by genetic mutations or environmental factors leads to the accumulation of Syt11 in midbrain DA neurons. This inhibits endocytosis and the following processes of vesicle replenishment, which then lead to the impairment of DA release and most probably the initiation of PD pathogenesis.

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Author contributions
ZZ., CW., and X.K. conceived the study and designed the experiments with the help of L.Zhou, and Z.Y. CW., X.K., L.Zhou, Z.C., Q.W., R.H., H.X., M.H., X.S., S.S., J.L., R.J., P.Z., and L.Zhang performed experiments and analyses. ZZ., CW., X.K., L.Zhou and Z.Y. wrote the manuscript.

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