Na\(^{+}/K^{+}\)-ATPase (NKA) plays important roles in maintaining cellular homeostasis. Conversely, reduced NKA activity has been reported in aging and neurodegenerative diseases. However, little is known about the function of NKA in the pathogenesis of Parkinson’s disease (PD). Here, we report that reduction of NKA activity in NKA\(^{1+/−}\) mice aggravates \(\alpha\)-synuclein–induced pathology, including a reduction in tyrosine hydroxylase (TH) and deficits in behavioral tests for memory, learning, and motor function. To reverse this effect, we generated an NKA-stabilizing monoclonal antibody, DR5-12D, against the DR region (\(^{887}\)DVEDSYGQQWTYEQR\(^{911}\)) of the NKA\(^{1}\) subunit. We demonstrate that DR5-12D can ameliorate \(\alpha\)-synuclein–induced TH loss and behavioral deficits by accelerating \(\alpha\)-synuclein degradation in neurons. The underlying mechanism for the beneficial effects of DR5-12D involves activation of NKA\(^{1}\)-dependent autophagy via increased AMPK/mTOR/ULK1 pathway signaling. Cumulatively, this work demonstrates that NKA activity is neuroprotective and that pharmacological activation of this pathway represents a new therapeutic strategy for PD.

### RESULTS

NKA\(^{1}\) deficiency aggravates PFF-induced pathology

To investigate the role of NKA\(^{1}\) in \(\alpha\)Syn-induced pathology, NKA\(^{1+/+}\) and NKA\(^{1+/−}\) mice were evaluated in the PFF model as previously described (fig. S1, A to E). PFF or phosphate-buffered saline (PBS) was injected into the striatum of mice 90 days before behavioral analysis (fig. S2A) with the Morris water maze (spatial learning and memory) and the rotarod test (neuromotor performance). After training for four consecutive days in the Morris water maze, NKA\(^{1+/−}\) mice treated with PFF exhibited a longer escape latency relative to PBS-treated controls. The same trend was also found in the NKA\(^{1+/−}\) mice (Fig. 1A and fig. S2B). These data confirm that PFF successfully induced PD-like neuronal injury.

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**INTRODUCTION**

Na\(^{+}/K^{+}\)–adenosine triphosphatase (NKA) is a transmembrane protein consisting of three subunits: \(\alpha\), \(\beta\), and \(\gamma\), with four isoforms of the catalytic \(\alpha\) subunit (\(\alpha 1\) to \(\alpha 4\)) (1). In the central nervous system, NKA requires about 40% of the energy delivered by respiration to maintain ion gradients across cell membranes (2). Recently, it has been reported that a progressive decline of NKA activity can exacerbate neurodegeneration in the aging process (3–8). Accumulating evidence also suggests a close relationship between NKA and Parkinson’s disease (PD). For example, clinical studies found that NKA activity was substantially reduced in erythrocytes of PD patients (9), and that motor symptoms of rapid-onset dystonia-Parkinsonism (RDP) and abnormal dopamine metabolites in cerebrospinal fluid (RDP) were found in patients harboring genetic mutations of \(\alpha\)Syn pathology through activation of Na\(^{+}/K^{+}\)-ATPase and autophagy-lysosome systems, and induction of mitochondrial dysfunction and endoplasmic reticulum stress. These defects cumulatively contribute to neuronal death and neurodegeneration (16). Therefore, increasing \(\alpha\)Syn degradation through autophagy may be an attractive target for PD therapy. Because NKA is a key regulator of autophagy (17–19), it is compelling to theorize that \(\alpha\)Syn clearance may be accelerated through activation of NKA-dependent autophagy. The extracellular region \(^{887}\)DVEDSYGQQWTYEQR\(^{911}\) (DR region), which is highly conserved among various NKA\(^{1}\) subunits, is the activation domain of NKA (20). Previously, our group developed a monoclonal antibody (DR-Ab) against the DR region of NKA that effectively activates NKA. DR-Ab protects against chronic heart failure and ischemic stroke injury both in vitro and in vivo (21–23). Given the reduction of NKA activity in PD and the importance of NKA in regulating autophagy, we hypothesize that activation of NKA by DR-Ab may be potentially protective against \(\alpha\)Syn pathology. To improve the specificity of DR-Ab in activating NKA and to facilitate subsequent drug development, we generated a monoclonal DR-Ab (DR5-12D) and studied its effect on \(\alpha\)Syn pathology. Intracerebral injection of preformed fibrils (PFFs) of \(\alpha\)Syn is a widely used rodent PD model due to its close replication of clinical symptoms observed in PD patients (24). Here, we demonstrate that reduction of NKA activity in NKA\(^{1}\) mice exacerbates PFF-induced pathological process, while DR5-12D alleviates PD-related pathology through activation of NKA-dependent autophagy to increase \(\alpha\)Syn clearance.

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**CELLULAR NEUROSCIENCE**

Anti–Na\(^{+}/K^{+}\)-ATPase immunotherapy ameliorates \(\alpha\)-synuclein pathology through activation of Na\(^{+}/K^{+}\)-ATPase \(\alpha 1\)-dependent autophagy

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Although no significant difference was found in the escape latency between NKA\(\alpha_{1}\)^{+/+} and NKA\(\alpha_{1}\)^{+/−} mice receiving only PBS treatment, a longer escape latency was found in the PFF-treated NKA\(\alpha_{1}\)^{+/−} mice compared to PFF-treated NKA\(\alpha_{1}\)^{+/+} mice (Fig. 1A). Representative swimming paths on training day 4 are shown in Fig. 1B. After removing the platform on probe test day, the swimming time in the target quadrant and the frequency to cross the platform zone were recorded (fig. S2C). These two indicators were decreased significantly in PFF-treated mice compared to PBS-treated controls (Fig. 1, C to E). Consistently, significant differences were found between the two genotypes of mice treated with PFF but not PBS. PFF-treated NKA\(\alpha_{1}\)^{+/−} mice had a shorter swimming time in the target quadrant and a lower frequency to cross the platform zone compared to those in PFF-treated NKA\(\alpha_{1}\)^{+/+} mice (Fig. 1, C to E). These data suggest that reduction of NKA\(\alpha_{1}\) exacerbates PFF-induced learning and memory impairment. Neuromotor performance evaluated by rotarod test confirmed that the latency to fall was decreased in PFF-treated mice compared to that in PBS-treated mice (Fig. 1F). Moreover, reduction of NKA\(\alpha_{1}\) further shortened the latency to fall in PFF-treated mice (Fig. 1F), indicating the essential role of NKA\(\alpha_{1}\) in neuromotor performance. Together, these results demonstrate that NKA\(\alpha_{1}\) deficiency contributes to PFF-induced behavioral signs.

We next examined whether NKA activity is altered in the PFF model. As shown in Fig. 2A, NKA activity was decreased in the PFF-treated wild-type (WT) mice and this effect was exacerbated by basal reduction of NKA\(\alpha_{1}\) in NKA\(\alpha_{1}\)^{+/−} mice. PFF-induced loss of dopaminergic axons in the striatum and dopaminergic neurons in the substantia nigra pars compacta (SNpc) was observed as determined by loss of tyrosine hydroxylase (TH) immunoreactivity, and these effects were exacerbated when NKA\(\alpha_{1}\) was reduced (Fig. 2, B and C). In addition, the relative accumulation of soluble \(\alpha\)Syn (TX-soluble fraction) versus insoluble \(\alpha\)Syn (SDS-soluble fraction) was examined. Insoluble \(\alpha\)Syn aggregates were detectable in both the striatum and midbrain regions upon PFF treatment, and this increased significantly in NKA\(\alpha_{1}\)^{+/−} mice compared to NKA\(\alpha_{1}\)^{+/+} mice (Fig. 2, D and E). Consistent with the \(\alpha\)Syn levels in SDS-soluble fractions, phosphorylated \(\alpha\)Syn (p-\(\alpha\)Syn, Ser129) was also significantly increased in the PFF-treated mice and this was further enhanced in both brain regions of NKA\(\alpha_{1}\)^{+/−} mice (Fig. 2, F and G). Immunostaining of the SNpc in the midbrain further confirmed that TH labeling was reduced in the PFF treatment groups, and this TH loss was exacerbated in NKA\(\alpha_{1}\)^{+/−} mice compared to that in NKA\(\alpha_{1}\)^{+/+} mice (Fig. 2H). Furthermore, p-\(\alpha\)Syn in the SNpc was increased with PFF treatment, and this increase was even greater in NKA\(\alpha_{1}\)^{+/−} mice than in WT mice.
Therefore, NKAα₁ deficiency, resulting in the reduction of NKA activity, may exacerbate PFF-induced pathological characteristics. To compare the contribution of NKA-dependent αSyn clearance in various brain cells, we determined αSyn levels in primary cultures of various brain cells in both WT and NKAα₁-deficient mice. A comparable reduction of NKAα₁ was found in neurons (40% of WT; fig. S3A), astrocytes (38% of WT; fig. S3C), and microglia (35% of WT; fig. S3E). The reduction of NKAα₁ also correlated with an increase in αSyn content that was similar in all cell types (neurons, fig. S3B; astrocytes, fig. S3D; and microglia, fig. S3F).
Monoclonal DR antibody ameliorates PFF-induced pathology

On the basis of our previous study that reported the development of an NKA-stabilizing polyclonal antibody (22), we generated a monoclonal DR antibody (DR-Ab) targeting the same DR region (897 DVEDSYGQWYTEQ911) of the NKAα subunit (Fig. 3A). The titer and specificity of DR-Ab clone DR5-12D were validated by enzyme-linked immunosorbent assay (ELISA) and Western blot analysis, respectively (Fig. 3, B and C). To examine the therapeutic effect of DR5-12D against PFF-induced injuries, DR5-12D or control immunoglobulin G (IgG) was administered intraperitoneally weekly from day 7 to day 90 after PFF/PBS injection (fig. S4A). Before the start of therapeutic experiments, blood-brain barrier penetration of DR antibody was validated by immunofluorescent staining of IgG in different brain regions. To minimize background labeling caused by endogenous mouse IgG, we used a rabbit polyclonal DR antibody to measure blood-brain barrier penetration. Conspicuous labeling was detected in brain regions including cortex, hippocampus, striatum, and midbrain 24 hours after DR antibody (intraperitoneal) treatment (fig. S4, B to E). In the Morris water maze test, on training days 3 and 4, the PFF-treated group had higher escape latency periods compared to PBS control, whereas the PFF + DR5-12D treatment group showed reduced latencies (Fig. 3D). Representative swimming paths on training day 4 are shown in Fig. 3E. On probe test day, the duration in the target quadrant and the frequency to cross the platform zone were significantly reduced in the PFF-treated group compared to PBS control, while these were increased by PFF + DR5-12D treatment (Fig. 3, F to H). Moreover, the PFF-treated group

Fig. 3. DR5-12D improves PFF-induced behavioral deficits. (A) Schematic illustration of NKAα subunit and DR region. (B) Titer of purified monoclonal DR5-12D examined by ELISA (n = 3). OD450, optical density at 450 nm. (C) Specificity of purified monoclonal DR5-12D detected by Western blotting analysis. Samples included purified NKA protein from the kidney of pigs and cell lysates extracted from SH-SY5Y cells and human embryonic kidney (HEK) 293 cells. BSA was provided as a negative control. (D) Escape latency time from training day 1 to day 4. (E) Representative swimming paths on training day 4. (F) Representative swimming paths on probe test day. (G) Duration presented as percentage of 60s in the target quadrant on probe test day. (H) Frequency to cross the platform zone on probe test day. (I) Latency to fall on three consecutive testing days in rotarod test. n = 11 in PBS group and n = 10 in PFF + IgG group and PFF + DR group. Values represent mean ± SEM. Two-way ANOVA followed by Bonferroni’s multiple comparisons test was used to analyze the data in (D) and (I). One-way ANOVA followed by Bonferroni’s multiple comparisons test was used to analyze other data.
had shorter latencies to fall compared to PBS control in the rotarod test, but this reduction was attenuated in the PFF + DR5-12D treatment group (Fig. 3I). These data indicate the therapeutic effect of DR5-12D on PFF-induced behavioral signs.

As anticipated, DR5-12D treatment attenuated NKA impairment in the PFF model (Fig. 4A). Concurrently, dopaminergic neuronal death was reduced by DR5-12D treatment as indicated by the restored expression of TH in both the striatum (Fig. 4B) and midbrain areas (Fig. 4C). Accumulation of αSyn in SDS-soluble fractions was markedly reduced in the DR5-12D treatment group compared with the IgG treatment group in both the striatum (Fig. 4D) and midbrain (Fig. 4E) regions. Similar results were found with respect to p-αSyn levels. DR5-12D treatment also markedly reduced PFF-induced up-regulation of p-αSyn in the above two brain regions (Fig. 4, F and G). Immunostaining of TH further confirmed the protective effect of DR5-12D against PFF-induced TH loss in the SNpc (Fig. 4H). In addition, p-αSyn level was also significantly reduced by DR5-12D treatment in the SNpc (Fig. 4H). Together, we conclude that DR5-12D...
has the potential to treat αSyn pathology, presumably by preservation of NKA activity.

**DR5-12D inhibits uptake and accelerates clearance of αSyn**

We further investigated the mechanism of the protective effect of DR5-12D on αSyn pathology in in vitro experiments. PFF was labeled with ATTO 488, a green fluorescent dye, for live-cell imaging (fig. S5A). Thereafter, the labeled PFF was detected by αSyn antibody to confirm the successful labeling, showing the merged signals (fig. S5B). These labeled PFFs were permeable to SH-SYSY cells and suitable for live-cell imaging (fig. S5C). One hour after DR5-12D treatment, SH-SYSY cells were treated with PFF–ATTO 488 for 15 min and then washed out. Compared to those in the IgG treatment group, reduced αSyn levels were observed in the DR5-12D treatment group at multiple time points after PFF–ATTO 488 treatment (Fig. 5A).

To exclude the possibility that the observed fluorescence was from extracellular αSyn fibrils adhering to the cell surface, trypsin-EDTA (0.01%) was used in all subsequent experiments to digest extracellular fibrils (25, 26). To determine the effect of DR5-12D on αSyn internalization, cells were pretreated with a lysosomal inhibitor [bafilomycin A1 (BA), 100 nM] for 1 hour to block αSyn degradation. After 1-hour treatment of DR5-12D or control IgG, PFFs were added into the cells for 0.5, 1, and 2 hours. As shown in Fig. 5B, αSyn was taken up by the cells 0.5 to 2 hours after PFF incubation in the IgG treatment group. This effect was attenuated by treatment with DR5-12D.

To observe the effect of the DR antibody on αSyn clearance, an equal amount of αSyn was loaded by treatment of cells with PFF for 1 hour followed by replacement of the medium with fresh medium containing DR5-12D. It was found that αSyn was significantly reduced with 24-hour treatment of DR5-12D compared to that with IgG treatment, indicating increased clearance of αSyn by DR5-12D treatment (Fig. 5C). Further, we validated this effect by Western blotting analysis. DR5-12D, but not IgG, treatment increased αSyn clearance in SDS-soluble fractions in SH-SYSY cells (Fig. 5D). In addition to the exogenous PFF treatment model, we also examined the effect of DR5-12D on αSyn clearance with an αSyn overexpression model. An αSyn–enhanced green fluorescent protein (eGFP) plasmid was transfected into SH-SYSY cells to generate an αSyn overexpression cell line, in which αSyn was detectable by Western blot (Fig. 5E). After 24-hour treatment with DR5-12D, the expression of αSyn was significantly decreased when compared to that of IgG treatment, suggesting the same effect of DR5-12D treatment as in the extracellular PFF model (Fig. 5F). Moreover, DR5-12D treatment also reduced endogenous expression of αSyn in primary neuronal culture (fig. S5D). Together, our data suggest that the protective effect of DR5-12D on αSyn pathology is mediated by both acceleration of clearance and inhibition of uptake of αSyn.

**NKA activity and NKAα1-dependent autophagy are required for DR5-12D–induced αSyn clearance**

Consistent with results obtained in the PFF mouse model, NKA activity was similarly decreased in the PFF cell model. SH-SYSY cells were pretreated with PFF for 1 hour and then washed out, followed by DR5-12D or control IgG treatment for 24 hours. The PFF-induced decrease in NKA activity was attenuated by DR5-12D but not by IgG treatment (Fig. 5G). Intriguingly, the effect of DR5-12D on αSyn clearance was blocked by pretreatment with 1 μM ouabain (OB; an NKA inhibitor) for 1 hour in both exogenous PFF (Fig. 5H) and αSyn overexpression models (Fig. 5I), confirming the essential role of NKA in the effect of DR5-12D on αSyn clearance. To further understand the mechanism underlying DR5-12D–accelerated αSyn clearance, we made use of inhibitors targeting the autophagy lysosomal system [BA and chloroquine (CQ)] and ubiquitin proteasome system [MG132 (MG)]. In the PFF model, inhibition of the autophagy lysosomal system with BA and CQ completely blocked the effect of DR5-12D on αSyn clearance, while MG treatment had no effect (Fig. 5J and fig. S6A). These data suggest an autophagy-dependent effect of DR5-12D on αSyn clearance that is independent of the ubiquitin proteasome pathway.

To confirm the role of NKAα1 in autophagy-dependent clearance of αSyn, we generated NKAα1 knockout (KO) Neuro2a cells with CRISPR-Cas9 technique (Fig. 5K). mRNA levels of autophagy-related genes including MAP1LC3B, SQSTM1, ULK1, BECN1, and ATG12 were all decreased in NKAα1 KO cells when compared to those of WT cells (Fig. 5L). In addition, while BA (100 nM) pretreatment significantly inhibited the degradation of LC3-II in WT cells, this effect was markedly attenuated in NKAα1 KO cells (Fig. 5M). Although DR5-12D treatment increased the accumulation of LC3-II in WT cells, this effect was absent in KO cells (Fig. 5M). Together, our data suggest that NKAα1-dependent autophagy is indispensable for αSyn clearance induced by DR5-12D treatment.

**DR5-12D activates AMPK/mTOR/ULK1 pathway in the PFF model**

We next investigated the effect of DR5-12D on autophagy-related signaling pathways in the PFF model. The 5’ adenosine monophosphate–activated protein kinase α (AMPKα)/mammalian target of rapamycin (mTOR)/ULK1 pathway, a positive regulator of autophagy, was first examined in SH-SYSY cells. After 1-hour PFF treatment, cells were washed with trypsin to remove extracellular PFF, which was then replaced by fresh media containing DR5-12D or control IgG for 24 hours. It was found that AMPK was markedly inhibited as reflected by the decreased ratio of AMP to ATP (adenosine triphosphate) (Fig. 6A) and the down-regulated expression of phospho-AMPKα (p-AMPKα; Thr172) (Fig. 6B). However, DR5-12D, but not IgG, attenuated the inhibitory effect of PFF on AMPK by increasing the ratio of AMP to ATP and up-regulating the expression of p-AMPKα (Fig. 6, A and B). Consistently, DR5-12D attenuated the altered expression of downstream signaling molecules including the increased phosphorylation of mTOR (p-mTOR; Ser2448) (Fig. 6C) and the decreased phosphorylation of ULK1 (p-ULK1, Ser555) (Fig. 6D). In addition, decreased expression of LC3-II was also attenuated by DR5-12D treatment (Fig. 6E). By contrast, interruption of this pathway using an AMPK inhibitor, compound C (20 μM), abrogated the effect of DR5-12D on αSyn clearance (Fig. 6F).

We also investigated another autophagy-related pathway, phosphatidylinositol 3-kinase (PI3K)/AKT pathway, which is a negative regulator of autophagy. In contrast to the marked effect of DR5-12D on AMPK, we failed to find any significant regulatory effect of DR5-12D on PI3K/AKT (fig. S6B). Together, we conclude that DR5-12D treatment increases αSyn clearance through activation of AMPK/mTOR/ULK1 pathway.

**DR5-12D inhibits the formation of NKAα1/AMPKα/αSyn complex**

Following the findings that NKAα1 was an essential regulator of autophagy and AMPK activation, we next explored the molecular interaction between NKAα1 and AMPKα. Co-immunoprecipitation (IP)
Fig. 5. DRS-12D increases αSyn clearance by maintaining NKA activity and activation of NKA α1-dependent autophagy. (A) Live-cell imaging showing the reduced αSyn level by DRS-12D treatment (n = 3; scale bar, 50 μm). (B) Effect of DRS-12D on PFF internalization (n = 4). SH-SY5Y cells were pretreated with BA for 1 hour. (C and D) Immunofluorescence staining and Western blotting analysis showing the increased clearance of αSyn by DRS-12D treatment (n = 4). Scale bars, 10 μm. (E and F) Representative Western blots showing the increased αSyn clearance by DRS-12D treatment in αSyn overexpression cells (n = 3). (G) Effect of DRS-12D on NKA activity (n = 4). (H and I) OB pretreatment blocked DRS-12D–accelerated αSyn clearance in both PFF-treated and αSyn overexpression cells [n = 5 in (H) and n = 4 in (I)]. (J) Effect of DRS-12D on αSyn clearance was blocked by lysosomal inhibitors (n = 4). (K) CRISPR-Cas9 technique knockout (KO) of NKA α1 in Neuro2a cells. (L) mRNA levels of autophagy-related genes in NKA α1 WT and KO cells (n = 6 to 8). (M) Effect of DRS-12D on LC3-II expression in NKA α1 WT and KO cells (n = 5). Two-way ANOVA was used to analyze the data in (B) and (M). Unpaired t test was used to analyze the data in (L). One-way ANOVA was used to analyze other data.
Fig. 6. DRS-12D treatment activates the AMPK/mTOR/ULK1 pathway and inhibits the formation of the NKAα1/AMPKα/αSyn complex in the PFF model. (A) DRS-12D significantly attenuated the PFF-suppressed ratio of AMP to ATP in the PFF model (n = 6). (B to D) Representative Western blots showing the expression of AMPKα and p-AMPKα (Thr172), mTOR and p-mTOR (Ser2448), and ULK1 and p-ULK1 (Ser555) in the PFF model [n = 4 in (B), n = 5 in (C), n = 4 in (D)]. (E) Representative Western blots showing the expression of LC3-II in the PFF model (n = 4). (F) Effect of DRS-12D on αSyn clearance was blocked by AMPK inhibitor (n = 4). (G and H) Co-IP analysis showing the interaction between NKAα1 and AMPKα in SH-SYSY cells in physiological state (n = 3). (I) DRS-12D treatment inhibited the formation of the NKAα1/AMPKα/αSyn complex in the PFF model (n = 4). (J) DRS-12D treatment reduced the expression of AMPKα and αSyn in the plasma membrane fractions (n = 4 to 6). One-way ANOVA was used for all the data analysis except for the data in (J), in which αSyn level was analyzed by unpaired t-test.
was used to examine the interplay between NKAα1 and AMPKα. In the normal physiological state, AMPKα was detected from the immunoprecipitate collected by anti-NKAα1 antibody treatment in SH-SY5Y cells (Fig. 6G). By contrast, NKAα1 was detected from anti-AMPKα antibody precipitated proteins (Fig. 6H), confirming a direct interaction between NKAα1 and AMPKα. In the PFF model, the expression of NKAα1 was increased in the immunoprecipitate collected by anti-AMPKα antibody treatment, suggesting the increased formation of the NKAα1/AMPKα complex. Meanwhile, αSyn was also detected in the same immunoprecipitate, suggesting the formation of a large NKAα1/AMPKα/αSyn complex. When treated with DR5-12D, the presence of NKAα1 and αSyn was decreased in the immunoprecipitate (Fig. 6I). Hence, we determined that addition of PFF increased the formation of the NKAα1/AMPKα complex and further contributed to the emergence of an NKAα1/AMPKα/αSyn complex, but DR5-12D treatment inhibited this complex formation. As NKAα1 distributes predominantly to the plasma membrane, while AMPKα mostly exists in cytosol, we investigated the process of complex formation by isolating plasma membrane fractions from total cell lysates to examine the presence of an NKAα1/AMPKα/αSyn complex. Intriguingly, PFF-induced accumulation of AMPKα and αSyn was observed in the plasma membrane fractions, and DR5-12D treatment reduced this membrane localization (Fig. 6J). To some extent, the above data suggest a process by which AMPKα, with the assistance of PFF, translocates from the cytosol to the plasma membrane to form a complex with NKAα1, while the addition of DR5-12D inhibits this complex formation (fig. S7).

**DISCUSSION**

Recent studies into the pathophysiology of PD have renewed our understanding of the function of the well-studied ion pump, NKA. Although clinical findings, such as a decreased NKA activity in erythrocytes of PD patients (9) and genetic mutations of ATP1A3 in RDP patients (10), have suggested that NKA may play a role in the pathogenesis of PD, the mechanisms underlying this process are poorly documented. A previous study on NKAα1 demonstrated that αSyn assemblies sequester NKAα1 to the plasma membrane, which leads to impaired NKA function (27). This study demonstrated the action of αSyn on reducing the pump function of NKA and characterized the importance of freely diffusing NKAα3 in maintaining NKA activity. Although α3 is a neuron-specific subunit of NKA, NKAα1 is expressed ubiquitously in all cells including neurons, and is essential for normal NKA activity (1). In our study, beyond addressing our knowledge gaps concerning the role of NKAα1 in PD pathogenesis, we aimed to develop a new therapeutic strategy based on maintaining NKA activity to treat PD.

To study the role of NKAα1 in αSyn pathology, NKAα1+/− and NKAα1+/+ mice were used in the PFF model followed by behavioral analyses. Consistent with previous findings, we found that PFF induced marked learning and memory deficits (28, 29). However, a slightly higher and earlier TH loss was found in this study compared to previous reports. The discrepancy may result from the different toxicities of PFF generated in different laboratories. Factors such as different species of αSyn, buffers, and experimental procedures (such as the sonication process) may cause different PFF toxicities (30). In addition, in the current study, we quantified the density of TH-positive cells rather than the total neuron count, which may also contribute to the discrepancy. Our study demonstrated that NKAα1 deficiency aggravates PFF-induced learning and memory impairment. Our evidence shows that PFF-induced neuromotor signs worsen due to the loss of NKAα1. In addition, PFF-induced TH loss was also markedly higher in NKAα1+/− mice when compared to that in NKAα1+/+ mice. This may be attributed to decreased NKA activity and increased pathogenic αSyn. We found that NKAα1 deficiency caused about a 20% reduction in NKA activity, while PFF reduced NKA activity in WT mice by 45%. These two manipulations seem to work synergistically to impair NKA activity. These data suggest that neurons with reduced NKA expression are more susceptible to PFF injury. This is similar to our previous findings that NKAα1-deficient mice are more susceptible to ischemic damage than WT mice (22).

On the basis of these findings, we hypothesized that maintaining NKA activity may be a new therapeutic strategy for αSyn pathology. To verify this hypothesis, we generated a monoclonal DR antibody (DR5-12D) that activates NKA. DR5-12D treatment alleviated the learning and memory impairment and improved neuromotor performance in the PFF model. Meanwhile, DR5-12D treatment, as expected, maintained NKA activity and further attenuated TH loss and reduced pathogenic αSyn. Hence, we confirm that DR5-12D protects against PFF-induced injuries through the preservation of NKA activity and the decrease of pathogenic αSyn.

We studied the role of NKA in αSyn regulation in the PFF model. Genetic reduction of NKAα1 enhanced, while DR5-12D treatment reduced, accumulation of insoluble, phosphorylated αSyn in the striatum and midbrain regions. These data suggest that NKA may play a role in the formation or clearance of αSyn aggregates. Our data in SH-SY5Y cells support the idea that NKA activity plays a role in clearance. Intriguingly, we found one species of αSyn oligomer around 50 kDa that responded significantly to the reduction of NKAα1 and DR treatment in the PFF model. We speculate that DR treatment preferentially elicits increased degradation efficiency for αSyn oligomers with high molecular weights relative to those with lower molecular weights. We also compared the role of NKAα1 in the regulation of αSyn among different brain cell types. No significant differences were found in αSyn accumulation in neurons, astrocytes, and microglia when NKAα1 expression was reduced in these cells. This suggests that NKAα1 is broadly important in the regulation of αSyn level in brain cells. Because αSyn aggregation induces dopaminergic neuronal injury, the present study therefore focused on studying how αSyn is cleared in neuronal cells.

It is expected that binding of DR5-12D to NKAα subunit activates NKA because the DR region has been reported to be the activation domain of NKA (20, 22). However, the potential mechanism by which DR5-12D reduces pathogenic αSyn is still elusive. We found in the present study that, in addition to reducing the uptake, DR-Ab accelerated αSyn clearance both in an exogenous PFF model and in an αSyn overexpression model at the cellular level. Inhibition of NKA activity using OB blocked the effect of DR5-12D on αSyn clearance, reaffirming an essential role of NKA activity in αSyn clearance. The ubiquitin-proteasome system and the autophagy-lysosome system are the two major systems for intracellular proteolysis, including αSyn degradation (12). Inhibition of the autophagy-lysosome system rather than the ubiquitin-proteasome system inhibited DR5-12D-induced αSyn clearance. Moreover, the effect of DR5-12D on increasing autophagic flux was blocked in NKAα1 KO cells. These data suggest the requirement of NKAα1-dependent autophagy for αSyn degradation with DR5-12D treatment.
Accumulating pathophysiological and genetic evidence has identified malfunctions of autophagy in PD (13–15). Impairment of the autophagy-lysosome system contributes to αSyn aggregation, which, in turn, inhibits autophagy, creating a positive feedback loop (31). In this study, we found that autophagic flux was inhibited by PFF treatment through the regulation of AMPK/mTOR/ULK1 pathway but not the PI3K/AKT pathway. There have been conflicting reports regarding the regulation of AMPK on NKA activity in different tissues. Studies have described positive regulation in skeletal muscle (32) and negative regulation in the lung (33), while little is known regarding this process in the brain. These controversial findings indicate a tissue-specific and complex relationship between AMPK and NKA. To further study the action of DR5-12D on activating the AMPK/mTOR/ULK1 pathway, we explored the molecular interaction between NKAα1 and AMPKα. Our data show that a direct interaction between NKAα1 and AMPKα exists and is enhanced by the formation of the NKAα1/AMPKα/αSyn complex in the PFF model, and that DR5-12D treatment inhibits this complex formation. In addition, we identify the process by which the NKAα1/AMPKα/αSyn complex forms. Our data suggest that αSyn is taken up into cells where it binds to cytosolic AMPKα and induces its translocation to the plasma membrane to form an NKAα1/AMPKα/αSyn complex. However, this does not exclude the possibility that extracellular αSyn may also induce complex formation through binding to NKAα1 at the cell surface. More studies are warranted to investigate how the complex is formed and how DR5-12D dissociates this complex through binding to NKAα1.

The action of αSyn on NKA activity is different between a previous study of NKAα3 and our current study of NKAα1. In light of the NKAα3 study, we understand that αSyn impairs NKA activity by trapping NKAα3 to form nanoclusters on the plasma membrane (27). The reduced NKA activity is attributed to the interaction between αSyn and the extracellular segment of NKAα3. Here, we revealed the intracellular events of αSyn. We found that αSyn interacts with AMPKα and contributes to the translocation of AMPKα to the plasma membrane to form a complex with NKAα1. The formation of the NKAα1/AMPKα/αSyn complex inhibits NKA activity and NKAα1-dependent autophagy.

In summary, our work uncovers the role of NKAα1 in αSyn pathology and explores the action of DR5-12D on αSyn clearance. However, more experiments are warranted to provide a comprehensive view of the importance of NKA in PD. Although we have demonstrated the effect of DR5-12D on αSyn clearance, it is possible that this effect may not be specific for αSyn. Future studies will address the specificity of DR5-12D treatment. In addition to the degradation process, a greater understanding of the mechanisms by which how NKA regulates αSyn internalization is needed as well. Moreover, exploration of NKA in different PD models, such as 6-hydroxydopamine–induced toxic model or genetic models, will deepen our understanding of the importance of NKA in PD. Together, our study not only broadens the potential function of NKA in PD but also sheds substantial light on developing new strategies for PD therapy.

MATERIALS AND METHODS
Preparation of αSyn PFFs
Human αSyn was expressed and purified as described previously (30). Human αSyn complementary DNA (cDNA) in bacterial expression plasmid pRK172 was a gift from M. Hasegawa (Tokyo Metropolitan Institute of Medical Science) (34). Briefly, αSyn-pRK172 plasmid was transformed and amplified in the BL21(DE3) Escherichia coli strain. Bacteria were collected thereafter, and pellets were resuspended in high-salt buffer [10 mM tris (pH 7.6), 750 mM NaCl, and 1 mM EDTA] containing a mixture of protease inhibitors. The cell lysate was sonicated and boiled to precipitate unwanted proteins. Then, the supernatant was collected and dialyzed with 10 mM tris (pH 7.6), 50 mM NaCl, and 1 mM EDTA. The supernatant was applied to a Hi-Trap Q HP anion-exchange column and eluted with a 0 to 0.5 M NaCl gradient (αSyn was eluted at 0.2 M NaCl). The elution was concentrated and dispersed with PBS buffer through 3.5-kDa MWCO Amicon Ultra Centrifuge filter devices (Millipore). Protein purity and identification were measured by Coomassie blue staining (fig. S1A) and Western blotting analysis (fig. S1B), respectively. For αSyn PFF formation, purified αSyn monomers [5 mg/ml, dissolved in 50 mM tris-HCl (pH 7.5) and 150 mM KCl] were incubated at 37°C for 7 days with continuous shaking at 1000 rpm in a thermomixer (Eppendorf, Germany). PFF formation was monitored by thioflavin T (Th-T) binding assay (fig. S1C). Aliquots were withdrawn from the assembly reactions daily and mixed with Th-T (10 μM) to measure the fluorescence at excitation at 440 nm and emissions at 480 nm. Further identification of PFF formation was observed by transmission electron microscopy (TEM). The structure of PFF before and after sonication was stained with phosphotungstic acid and observed by TEM (fig. S1D). Western blotting was also used to confirm the formation of PFF (fig. S1E). All the PFFs were sonicated into small fractions by a Sonic dismembrator system (Thermo Fisher Scientific, catalog no. FB120110) with a 0.16-inch microtip at 60 pulses and 10% power (total of 30 and 0.5 s on and 0.5 s off) before applying to experiments.

PFF dissolved in PBS was administered at 5 μg per mouse in animal experiments and 2 μg/ml in cell experiments. In cell experiments, trypsin-EDTA was used to digest the remaining extracellular fibrils after PFF treatment. Cells were washed three times with PBS and incubated with trypsin-EDTA (0.01%) for 1 min at 37°C to remove extracellular αSyn, followed by a wash with Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum to stop the trypsinization.

Generation of anti-NKA monoclonal antibody
The antigen, DR peptide (DVEDSYGQQWTYEQR) (1st Base, Singapore), was conjugated with keyhole limpet hemocyanin (KLH). BALB/c mice (female, 6 to 8 weeks old) were immunized three times intraperitoneally with DR peptide–KLH every 2 weeks. The initial dose of DR peptide–KLH was 100 μg emulsified in complete Freund’s adjuvant (Sigma-Aldrich, F5881), followed by two injections of DR peptide–KLH (50 μg) emulsified with incomplete Freund’s adjuvant (Sigma-Aldrich, F5506). Splenocytes were isolated from the immunized mice 3 days after the last immunization for hybridoma production. Briefly, splenocytes were fused with SP2/0 myeloma cells at the ratio of 4:1 using 50% (v/v) polyethylene glycol. Complete RPMI containing 20% fetal calf serum and hypoxanthine-thymidine were used for the hybridoma cell culture. Antibody production was measured by ELISA in the supernatant of the cultured hybridomas. Positive hybridomas were picked and cloned by limited dilution. Further production of the monoclonal antibody was conducted by collecting ascites from BALB/c mice (female, 6 to 8 weeks old). Typically, pristane (0.5 ml per mouse, Sigma-Aldrich, P2870) was injected into the peritoneum of the mice. After 7 days, hybridomas (5 × 10⁶ per mouse, etc.) were cultured with RPMI and 10% fetal calf serum.
intrapitoneally) were injected into the mice to produce monoclonal antibody. Ascites was collected 10 to 14 days later for monoclonal antibody purification. Protein A/G spin columns (Thermo Fisher Scientific, #89962) were used to purify the monoclonal antibody from ascites according to the manufacturer’s instruction. The titer of the purified DR antibody was measured by ELISA, while the specificity was validated by Western blotting using NKA protein purified from the kidney of pigs as described (35) and cell lysates extracted from SH-SY5Y cells and human embryonic kidney (HEK) 293 cells. In cell experiments, monoclonal DR antibody (40 μg/ml) and control IgG (40 μg/ml) were administered. In animal experiments, monoclonal DR antibody (30 mg/kg) and control IgG (30 mg/kg) were administrated.

### Stereotaxic injections

NKAα1+/− mice were generated and provided by J. B. Lingrel in the University of Cincinnati, USA (36). The NKAα1+/− and NKAα1−/− mice were backcrossed with C57BL/6. Breeding and housing were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the National University of Singapore Institutional Animal Care and Use Committee. Intracerebral injection of PFF was performed as previously described (37). Briefly, after anesthesia, 3-month-old male NKAα1+/− and NKAα1−/− C57BL/6 mice were injected with PFF (5 μg per mouse) stereotaxically, and the mice in control group were injected with PBS. A single needle insertion into the right striatum (coordinates: +0.2 mm to bregma, 2.0 mm from midline, 2.6 mm below the dura) was applied via a Hamilton syringe (0.1 μl/min, 2.5 μl). After surgery, animals were monitored, and postsurgical care was provided. Monoclonal anti-NKA antibody (DR) (30 mg/kg) and control IgG (30 mg/kg) were injected (intraperitoneally, weekly) 7 days later after PFF injection. The treatment was terminated by day 90 after PFF injection.

### Morris water maze

Morris water maze test was performed 90 days after PFF injection. Before testing, mice were subjected to swim for 60 s and stand on the platform for 10 s to familiarize the test apparatus. During the testing days from day 1 to day 4, the location of the platform remained constant. Mice that did not find the platform were guided to the platform and given a latency score of 60 s. Mice that found the platform were permitted to stay on the platform for 10 s. Each mouse was tested for two sessions with an intertrial interval of 15 min. The average time to find the platform and the swimming path were recorded. On day 5, a probe test was conducted that each mouse was tested to search for the platform for 60 s in the absence of the platform. Each mouse was tested for two sessions with an intertrial interval of 15 min. The average time consumed in the quadrant that had the platform previously and the number to across the platform area were recorded.

### Rotarod test

The rotarod test was performed after the Morris water maze test. During the training phase for two consecutive days, each mouse was placed on the rotarod at a constant speed of 12 rpm for a maximum time of 120 s. While on the three consecutive testing days, mice were placed on the rotarod with an accelerating mode (4 to 40 rpm in 5 min). Mice were given two trials with an intertrial interval of 60 min. The maximum time for each session was 5 min, and the average time spent on the rotarod was recorded.

### Primary cultures

Primary neurons were prepared from E17 NKAα1+/+ and NKAα1−/− mice as previously described (22). Briefly, the midbrain was carefully dissected in ice-cold PBS and dissociated using trypsin (0.25%, 12 min at 37°C). Neurons were plated onto poly-d-lysine–treated culture plates in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were incubated in a 5% CO2 incubator at 37°C for 4 hours, after which the media were replaced with serum-free Neurobasal/B27/glutamine media. Half of the medium was exchanged every 3 days. All experiments were performed on neurons that were cultured for 12 to 14 days in vitro.

Primary astrocytes were isolated as described previously (38). Briefly, the midbrain of neonatal mice was separated from meninges and basal ganglia. Tissues were dissociated with 0.25% trypsin at 37°C and terminated by DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were plated on flasks, and the culture medium was replaced with fresh medium 24 hours later. Then, media were replaced every 3 days. After culturing for 14 days, microglia were detached from the astrocytes by shaking flasks at 200 rpm for 24 hours at 37°C on a shaker. Detached microglia were plated and incubated for 30 min at 37°C and 5% CO2. Unbound cells were removed by changing the culture medium.

### Western blotting analysis

Dissected brain regions or the cultured cells were prepared for sequential extraction of proteins. Samples were lysed in TX-soluble buffer [1% Triton X-100, 150 mM NaCl, 50 mM tris (pH 8.0), and protease inhibitors] to extract TX-soluble fractions. The insoluble pellet was resuspended in SDS-soluble buffer [2% SDS, 1% Triton X-100, 150 mM NaCl, 50 mM tris (pH 8.0), and protease inhibitors] and sonicated into SDS-soluble fractions. Protein concentration was measured with a BCA kit (Pierce, USA). Samples were separated on SDS–polyacrylamide gel electrophoresis gels (8 to 15%) and transferred onto polyvinylidene difluoride membranes. After blocking in 5% nonfat milk in TBST buffer (10 mM tris-HCl, 120 mM NaCl, and 0.1% Tween 20, pH 7.4) at room temperature for 1 hour, the membranes were probed with various primary antibodies at 4°C overnight. Membranes were then washed in TBST and incubated with appropriate secondary antibodies at room temperature for 1 hour. After washing in TBST, the target antigens on the blots were visualized by ECL substrate in a ChemiDoc XRS system (Bio-Rad). Band density was quantified by densitometry analysis of the scanned blots using ImageJ. Primary and secondary antibodies used in this study were listed in table S1.

### Co-IP assay

Cell samples were harvested with lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM tris, pH 8.0) containing protease inhibitors. Equal amounts of protein were then incubated with anti-AMPKα antibody or anti-NKAα1 antibody at 4°C overnight. Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, SC-2003) was added to incubate with samples for 4 hours at room temperature. The IP complexes were washed three times with lysis buffer and denatured by adding 2x Laemmli sample buffer, followed by boiling for 5 min. Western blotting analysis was used to detect the IP complexes further.

### Immunofluorescence assay

Brain slices (20 μm) and cell samples were fixed in 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100. After blocking...
in 5% bovine serum albumin (BSA) in PBS buffer at room temperature for 1 hour, the samples were probed with various primary antibodies at 4°C overnight. After washing three times with PBS, samples were incubated with fluorescent secondary antibodies for 2 hours at room temperature before they were mounted with 4',6-diamidino-2-phenylindole (DAPI)–containing mounting medium (Invitrogen, Carlsbad, CA, USA). Photos were taken using a fluorescence microscope (Nikon, Japan). ImageJ was used for the assessment of TH-positive SNpc neurons. Every sixth coronal frozen section was collected (20 μm per section) from SNpc and stained by immunofluorescence with anti-TH antibody. For each mouse, we examined and analyzed six sections. Images were converted to grayscale and thresholded using ImageJ. The same thresholds were applied to each biological and technical replicate. Area occupied in each image was analyzed by the “Analyze particle” function in ImageJ. The intensity of αSyn, p-αSyn, and lysosomal substrate was analyzed by ImageJ.

Biocytin labeling of plasma membrane protein

Plasma membrane proteins were labeled with SULFO-NHS-SS-biotin (1 mg/ml, Pierce, USA) for 1 hour. Cells were then rinsed with PBS containing 100 mM glycine thoroughly to quench unreacted biotin (1 mg/ml, Pierce, USA) for 1 hour. Cells were then rinsed with PBS and mounted on slides (Fisher Scientific). Photos were taken using a fluorescence microscope (Nikon, Japan). ImageJ was used for the assessment of the positive plasma membrane proteins. Each image was analyzed by the “Analyze particle” function in ImageJ. The number of plasma membrane proteins was determined by the intensity of the positive plasma membrane proteins.

NKA activity assay

Brain and cell samples were lysed in buffer A (20 mM Heps, 250 mM sucrose, 2 mM EDTA, and 1 mM MgCl₂, pH 7.4). The pellet was resuspended in buffer A, and the protein concentration was determined with a BCA assay kit (Pierce, USA). Samples were divided into two aliquots: One aliquot (50 μl) was incubated with reaction buffer 1 [50 μl, 200 mM tris (pH 7.5), 30 mM MgCl₂, 200 mM NaCl, 60 mM KCl, and 10 mM EGTA], and the other aliquot (50 μl) was incubated with reaction buffer 2 (buffer 1 + 1 mM OB; Sigma-Aldrich, O3125). ATP (1 mM) was added to start the reactions at 37°C for 10 min. After terminating the reactions by adding trichloroacetic acid [10 μl, 100% (w/v)], samples were placed on ice for 1 hour. Free phosphates were then collected in the supernatant of the samples after centrifuging at 20,000g for 30 min. Phosphatase colorimetric kit (Sigma-Aldrich, MAK030) was used to measure the free phosphates. The enzyme activity of NKA was defined as the difference of absorbance at 650 nm between the two aliquots.

Live-cell imaging

An ATTO 488 protein labeling kit (Sigma-Aldrich, 38371) was used to label PFF. Hoechst 33342 (Thermo Fisher Scientific, 62249) was used to stain the nuclei of the cells. SH-SY5Y cells were pretreated with DR5-12D for 1 hour followed by PFF–ATTO 488 treatment. The cells were replaced with fresh medium 15 min after PFF–ATTO 488 treatment. Live images were recorded every 15 min for 1 hour using a Leica fully motorized inverted microscope (Leica, DMi8).

Generation of stable cell lines

To generate αSyn overexpression cell line, αSyn-eGFP plasmid (Addgene, #40822) was transfected into SH-SY5Y cells using a Lipofectamine 3000 transfection kit (Invitrogen, L3000015). Successfully transfected cells were selected by kanamycin, and the expression of αSyn was validated by Western blotting.

NKAα1 CRISPR-Cas9 KO plasmid (Santa Cruz Biotechnology, SC-419236) was used to generate NKAα1 KO stable cell line in Neuro2a cells. Briefly, cells were transfected with NKAα1 CRISPR-Cas9 KO plasmid and homology-directed repair plasmid, which is used to repair site-specific double-strand breaks (DSBs) caused by KO plasmids. After DSB and subsequent repairment, the cells incorporated puromycin resistance and red fluorescent protein (RFP) for selection. Western blotting was used to monitor the KO efficiency of NKAα1.

Reverse transcription quantitative polymerase chain reaction

Total RNA extracted from Neuro2a cells was used to generate cDNA by a reverse transcription kit (Promega, A5001). SYBR Green–based (Promega, A6001) real-time polymerase chain reaction was performed to quantify mRNA levels. Primers used in this study were listed in table S2.

Measurement of AMP and ATP levels

Cellular AMP and ATP levels were measured using an AMP kit (Promega, V5011) and an ATP kit (Promega, G9242) according to the manufacturer’s instructions, respectively. The ratio of AMP to ATP was analyzed.

Statistical analysis

Unpaired Student’s t test was used to compare one variable between two groups. One-way analysis of variance (ANOVA) was used to compare one variable in three or more groups followed by Bonferroni’s multiple comparisons test. Two-way ANOVA was used to compare two independent variables followed by Bonferroni’s multiple comparisons test. GraphPad Prism 7 software was used for analysis. Graphs showed the values by mean ± SEM, with individual data points. P < 0.05 was predetermined as the threshold for statistical significance. All the data were collected by more than three biological replicates with two technical replicates in in vitro experiments, and detailed information was presented in figure legends.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/5/eabc5062/DC1

View/request a protocol for this paper from Bio-protocol.

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reciprocal relationship. patients with 
R. Alzheimer patient amyloid- 
A. Alzheimer’s disease. 
S. some soluble enzymes related to 
autophagy triggers preferential cell death of 
alpha-synuclein in 
fibric β-synuclein models advance our understanding 
alpha-synuclein pathology through activation of 
α-synucleinopathy in 
D2 receptor restricts astrocytic NLRP3 inflammasome activation via enhancing 
Na+ gradient. Important forms related to 
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Acknowledgments: We thank J. B. Lingel for the gift of NAAα1-13C5– mice and D. Ramond Herr for critical reading of the manuscript. Funding: This work was supported by the Singapore National Medical Research Council (NMRC/CIRG/1432/2013), Ministry of Education of Singapore Tier 2 Research grant (MOE2017-T2-2-029), and National Nature Science Foundation of China (NSFC 18172865). Author contributions: J.-S.B. and L.C. conceived this project. L.C. performed most experiments, analyzed the data, and wrote the manuscript. S.X. generated the monoclonal DR5-12D and two stable cell lines. L.D., Y.Z., Z.W., and H.S. helped with data analysis. M.Z., W.T.L., and X.N. provided critical ideas. J.-S.B. supervised all the experimental procedures and revised the manuscript. Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Submitted 29 April 2020
Accepted 9 December 2020
Published 27 January 2021
10.1126/sciadv.abc5062

Citation: L. Cao, S. Xiong, Z. Wu, L. Ding, Y. Zhou, H. Sun, M. Zhu, W. T. Lee, X. Nie, J.-S. Bian, Anti–Na"/K"-ATPase immunotherapy ameliorates α-synuclein pathology through activation of Na"/K"-ATPase α1-dependent autophagy. Sci. Adv. 7, eabc5062 (2021).
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Sci Adv 7 (5), eabc5062.
DOI: 10.1126/sciadv.eabc5062