α-Synuclein binds and sequesters PIKE-L into Lewy bodies, triggering dopaminergic cell death via AMPK hyperactivation

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The abnormal aggregation of fibrillar α-synuclein in Lewy bodies plays a critical role in the pathogenesis of Parkinson's disease. However, the molecular mechanisms regulating α-synuclein pathological effects are incompletely understood. Here we show that α-synuclein binds phosphoinositide-3 kinase enhancer L (PIKE-L) in a phosphorylation-dependent manner and sequesters it in Lewy bodies, leading to dopaminergic cell death via AMP-activated protein kinase (AMPK) hyperactivation. α-Synuclein interacts with PIKE-L, an AMPK inhibitory binding partner, and this action is increased by S129 phosphorylation through AMPK and is decreased by Y125 phosphorylation via Src family kinase Fyn. A pleckstrin homology (PH) domain in PIKE-L directly binds α-synuclein and antagonizes its aggregation. Accordingly, PIKE-L overexpression decreases dopaminergic cell death elicited by 1-methyl-4-phenylpyridinium (MPP^+), whereas PIKE-L knockdown elevates α-synuclein oligomerization and cell death. The overexpression of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or α-synuclein induces greater dopaminergic cell loss and more severe motor defects in PIKE-KO and Fyn-KO mice than in wild-type mice, and these effects are attenuated by the expression of dominant-negative AMPK. Hence, our findings demonstrate that α-synuclein neutralizes PIKE-L's neuroprotective actions in synucleinopathies, triggering dopaminergic neuronal death by hyperactivating AMPK.

neurodegenerative disease | dopamine | Lewy bodies

Parkinson's disease (PD), the second most prevalent age-related neurodegenerative disease, is characterized by progressive selective loss of dopaminergic (DAergic) neurons in the substantia nigra pars compacta with the concomitant loss of nigrostriatal DAergic termini and the resulting motor symptoms. Altered protein folding is thought to play a key role in the etiopathogenesis of PD, because the disorder is characterized neuropathologically by the accumulation of intraneuronal protein aggregates (Lewy bodies). The principal component of the Lewy body is aggregated α-synuclein (1). Point mutations in the α-synuclein gene (*SNCA*) cause rare familial forms of PD. Importantly, multiplication of wild-type *SNCA* also causes a familial form of PD, indicating that an increased level of normal α-synuclein protein is sufficient to cause the disease. The Lewy bodies stain positively for α-synuclein, ubiquitin, and a specific form of posttranslationally modified α-synuclein that is phosphorylated on S129 and is found only in Lewy bodies and Lewy neurites. The C terminus of α-synuclein can be phosphorylated at Y125 and at S129 by Src family kinase Fyn and various casein kinases or AMP-activated protein kinase (AMPK), respectively (2–5). It has been proposed that p-Y125 and p-S129 have opposing effects on neurotoxicity and soluble oligomer formation. α-Synuclein neurotoxicity in PD may result from an imbalance between the detrimental, oligomer-promoting effect of p-S129 and a neuroprotective action of p-Y125 that inhibits toxic oligomer formation (6). AMPK consists of α, β, and γ subunits. The α subunit possesses catalytic activity. Phosphorylation of the Thr residue at 172 in the α subunit is essential for AMPK activation to function as a protein kinase (7). AMPK is a key sensor of cellular energy status. AMPK signaling regulates the energy balance at the cellular, organ, and whole-body level. AMPK activation may have dual functions in the regulation of neuronal survival and death: AMPK provides a protective effect during transient energy depletion, as exemplified in a model of neuronal Ca^{2+} overloading. Conversely, prolonged AMPK activation can lead to neuronal cell death (8). AMPK activation is commonly present in many neurological diseases, including stroke (9), Huntington's disease (10), Alzheimer's disease (11), and synucleinopathies (5). Lactate levels are increased in the aging brain (12), in PD-affected subjects as compared with age-matched controls (13), and in mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (14). Recently, it has been reported that lactic acid up-regulates the activity of AMPK (15), leading to α-synuclein accumulation and oligomerization via AMPK phosphorylation of S129 in a time- and concentration-dependent manner (5). Phosphatidylinositol 3-kinase enhancers (PIKEs) are a family of GTPases that participate in multiple cellular processes including cell survival, brain development, memory formation, and metabolism (16–18). In the CNS, phosphoinositide-3 kinase enhancer L (PIKE-L) is highly enriched in the nerve termini (19–21) where it interacts with various receptors to trigger PI3K activation and displays neuroprotective activities (21, 22). In addition, PIKE-L exerts neuroprotective actions by protecting the DNase inhibitor SET from degradation by asparagine endopeptidase during stroke or kainic acid treatment (23). Interestingly,

Significance

We discovered that α-synuclein interacts with the neuroprotective protein phosphoinositide-3 kinase enhancer L (PIKE-L) in an S129 phosphorylation-dependent manner and sequesters PIKE-L in Lewy bodies, leading to the hyperactivation of AMP-activated protein kinase (AMPK) and subsequent dopaminergic neuronal cell death. Our findings may identify a molecular mechanism by which α-synuclein triggers dopaminergic neuronal cell death in Parkinson's disease.

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PIKE-L is important in regulating the development of the neocortex (24) and also is implicated in brain-derived neurotrophic factor (BDNF)/TrkB signaling cascades. The BDNF-mediated PI3K/Akt pathway, but not the MAPK pathway, is selectively diminished when PIKE is depleted. Consequently, PIKE$^{-/-}$ neurons are more vulnerable to glutamate- or stroke-induced cell death (24–26). Most recently, we demonstrated that PIKE-A, an isoform in the PIKE family, binds the AMPK α subunit and suppresses its activation and kinase activity, and this interaction is enhanced by Fyn phosphorylation of PIKE-A (27). In the current study, we report that α-synuclein associates with PIKE-L, which is regulated by p-S129 and p-Y125, which in turn are mediated by AMPK and Fyn, respectively. This interaction prevents α-synuclein aggregation and blocks its neurotoxic effect. Using both 1-methyl-4-phenylpyridinium (MPP$^+$) neurotoxin and α-synuclein genetic models of nigrostriatal degeneration, we demonstrate that PIKE-L and Fyn are required to prevent DAergic cell loss from both toxic stimuli and that inhibition of AMPK rescues DAergic cell death triggered by MPTP. This finding may provide insight into the molecular mechanism by which α-synuclein exerts its neurotoxic effects in DAergic neurons and may shed important light on the etiology of PD.

Results

α-Synuclein Binds PIKE-L in an S129 Phosphorylation-Dependent Manner. To explore whether PIKE-L is implicated in DAergic neuronal survival, we monitored dopamine (DA) metabolism in the substantia nigra, striatum, and hippocampus of wild-type mice and age-matched PIKE-KO littermates. DA is primarily oxidized by monoamine oxidase B (MAO-B) into the metabolite 3,4-dihydroxyphenylacetic acid (DOPAC). HPLC analysis revealed that striatal DA and DOPAC did not differ in 3-m-old wild-type and PIKE$^{-/-}$ mice. By contrast, DA levels in the substantia nigra and hippocampus in were significantly reduced 3- and 8-mo-old PIKE$^{-/-}$ mice (Fig. S1), indicating that DA metabolism is altered when PIKE is knocked out. Hence, PIKE might be involved in PD progression. Because lactate activates AMPK, which promotes phosphorylation of α-synuclein on S129, leading to its aggregation and neuritis reduction (5), we hypothesized that there might be crosstalk between PIKE and α-synuclein. Coimmunoprecipitation showed that α-synuclein interacts with PIKE-L upon activation of AMPK via 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) or metformin treatment, whereas the AMPK inhibitor compound C blocks this interaction (Fig. 1*A*, *Top*). Phosphorylation of AMPK and α-synuclein (S129) correlated with the binding affinities between PIKE-L and α-synuclein (Fig. 1*A*, *Third and Bottom*). As expected, the nonphosphorylatable S129A mutant barely interacted with PIKE-L regardless of AMPK activation status (Fig. 1*B*), indicating that S129 phosphorylation is required for α-synuclein to associate with PIKE-L. Consequently, constitutively active AMPK (AMPK-CA) strongly phosphorylated α-synuclein on S129, resulting in increased association with PIKE-L, compared with control or kinase-dead AMPK (AMP-KD) (Fig. 1*C*). Accordingly, the phosphorylation mimetic mutant α-synuclein S129D demonstrated a strong interaction with PIKE-L, but S129A did not bind PIKE-L to any significant degree (Fig. 1*D*), suggesting that S129 phosphorylation is indispensable for the association between α-synuclein and PIKE-L. Coimmunoprecipitation revealed that endogenous PIKE-L and α-synuclein interact with each other specifically in the brain (Fig. 1*E*). Finally, to assess whether PIKE-L interacts with α-synuclein in the brains of humans with PD, we conducted dual-label immunohistochemical staining and found that PIKE and α-synuclein colocalized in Lewy body inclusions (Fig. 1*F*), indicating that they might bind to each other in the Lewy bodies and that PIKE-L might be implicated in PD etiology via interaction with α-synuclein. A truncation assay revealed that the PIKE-L C-terminal fragment comprising amino acids 900–1,186 is not required for α-synuclein binding (Fig. S2*A*). Fragmenting PIKE-L further into different functional domains, we found that the pleckstrin homology (PH) domain in PIKE-L interacted directly with α-synuclein in a GST pull-down assay (Fig. S2*B*). An in vitro aggregation assay

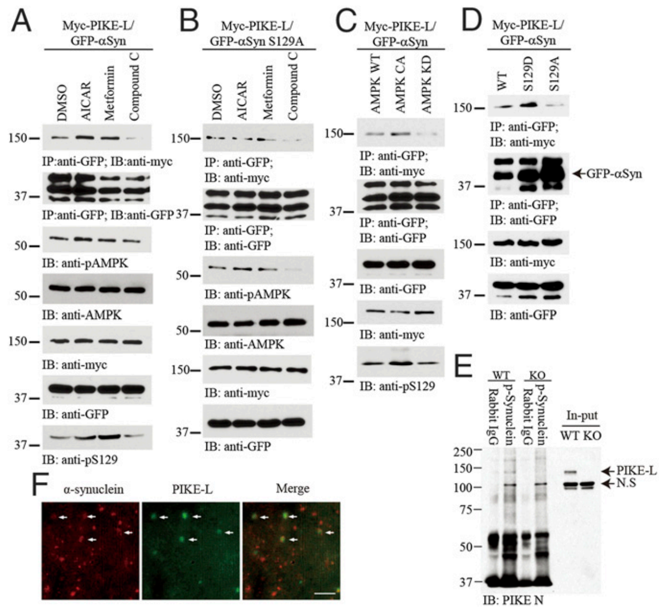

Fig. 1. α-Synuclein binds PIKE-L in an S129 phosphorylation-dependent manner. (*A*) AMPK mediates the interaction between PIKE-L and α-synuclein. HEK 293 cells were cotransfected with Myc–PIKE-L and GFP–α-synuclein and were treated with AICAR (200 μM), metformin (1 mM), or compound C (200 nM) for 24 h. The binding between PIKE-L and α-synuclein was confirmed using immunoprecipitation. (*B*) α-Synuclein S129 phosphorylation is required for α-synuclein to bind PIKE-L. HEK 293 cells were cotransfected with Myc–PIKE-L and GFP–α-synuclein S129A and were treated with AICAR, metformin, or compound C. The binding between PIKE-L and S129A was confirmed using immunoprecipitation. The expression of AMPK, transfected PIKE-L, and α-synuclein was examined also. (*C*) AMPK-CA elevates the PIKE-L/α-synuclein interaction. Myc-PIKE-L and GFP-α-synuclein were cotransfected to HEK 293 cells with wild-type AMPK, AMPK-CA, or AMPK-KD. (*D*) S129 phosphorylation of α-synuclein is required for the binding of α-synuclein to PIKE-L. Myc-PIKE-L was cotransfected to HEK 293 cells with GFP–α-synuclein wild-type, S129D, or S129A. The binding of these proteins was examined by immunoprecipitation. (*E*) Endogenous PIKE-L binds α-synuclein in mouse brain. Immunoprecipitation and immunoblotting were performed in substantia nigra lysates of wild-type and PIKE-KO mice. The interaction of α-synuclein (S129) and PIKE-L in the brain was confirmed. (*F*) PIKE-L/α-synuclein colocalization in Lewy bodies. PIKE-L (green) was stained with α-synuclein (red) in cortex from normal controls and patients with PD. PIKE-L and α-synuclein were colocalized in Lewy bodies (arrows). (Scale bar, 100 μm.)

demonstrated that the PH domain significantly reduced the aggregation of both wild-type α-synuclein and the A53T mutant as measured by a Thioflavin T aggregation assay (Fig. S2*C*). Ultracentrifugation also showed that the presence of the PH domain reduces the degree of aggregated α-synuclein in the pellet, resulting in the augmentation of soluble α-synuclein (Fig. S2*D*). EM analysis validated the fibrillization of recombinant α-synuclein proteins (Fig. S2*E*). Hence, these studies indicate that the PIKE-L PH domain binds α-synuclein and inhibits its aggregation. Phosphorylation of α-synuclein on amino acids Y125 and S129 produces opposing effects (6). Accordingly, we wanted to test whether Fyn phosphorylation of Y125 also regulates the binding activity of α-synuclein with PIKE-L. Coimmunoprecipitation revealed that constitutively active Fyn (Fyn-CA) strongly phosphorylated α-synuclein Y125, abolishing the association between PIKE-L and α-synuclein (Fig. S3*A*). When Fyn was inhibited by Src kinase inhibitor (PP2), but not by the inactive analog of PP2 (PP3), α-synuclein binding to PIKE-L was elevated (Fig. S3*B*, first blot). As expected, α-synuclein Y125 phosphorylation was potently blocked by PP2 but not by PP3 (Fig. S3*B*, sixth blot), indicating that Fyn-mediated Y125 phosphorylation inhibits the association between PIKE-L and α-synuclein. These findings also were confirmed in vivo (Fig. S3*C*). Thus, Fyn phosphorylates α-synuclein Y125 and blocks its binding to PIKE-L.

α-Synuclein Overexpression Strips PIKE-L from AMPK, Inducing AMPK Activation and Cell Death. To explore whether α-synuclein overexpression affects the association between PIKE-L and AMPK, we performed a competition assay. We found that the association between PIKE-L and AMPK was gradually repressed as the concentration of α-synuclein was progressively increased (Fig. 2*A*, *Top*). Notably, AMPK T172 phosphorylation was steadily increased in an α-synuclein dose-dependent manner (Fig. 2*A*, *Fourth and Fifth*), whereas PIKE-L overexpression suppressed its phosphorylation. Consequently, knockdown of endogenous PIKE by shRNA expression elicited additional AMPK T172 phosphorylation and its downstream signaling, Acetyl-CoA carboxylase phosphorylation (p-ACC), tightly coupled with p-AMPK patterns (Fig. 2*B*). As a major energy sensor in cells, AMPK activation is intimately regulated by the cellular AMP/ATP ratio. Overexpression of α-synuclein elevates the cellular ADP/ATP ratio, presumably because of α-synuclein's ability to modulate mitochondrial function. This ratio was reduced by PIKE-L overexpression. Knockdown of endogenous PIKE-L led to further escalation of ADP/ATP ratios (Fig. 2*C*). A cell-death assay in which lactate dehydrogenase (LDH) was released in the medium showed similar patterns, demonstrating that α-synuclein overexpression induced DAergic cell death, which was attenuated by concomitant PIKE-L overexpression. Finally, PIKE-L knockdown further enhanced the neurodegenerative effect of α-synuclein (Fig. 2*D*). Lactate induces AMPK activation and α-synuclein S129 phosphorylation, leading to its aggregation and neurite reduction (5). The neurotoxicity of α-synuclein is related to its oligomerization or fibrillization. To assess the effect of PIKE-L on α-synuclein aggregation, we transfected SH-SY5Y cells with PIKE-L plasmid or silenced PIKE-L expression using shRNA, followed by infection with a virus expressing α-synuclein. Next, we treated the cells with vehicle or 20 mM lactate for 3 d. Lactate treatment strongly induced AMPK pathway activation (p-T172 and p-ACC), which was blocked by PIKE-L overexpression, whereas knockdown of PIKE-L resulted in further activation of AMPK. Noticeably, lactate triggered α-synuclein aggregation, which was exacerbated in the PIKE-L–depleted cells, whereas overexpression of PIKE-L reduced α-synuclein aggregation (Fig. 2*E*), in keeping with our in vitro observations that the PIKE-L PH domain inhibits α-synuclein aggregation (Fig. S2 *D* and *E*). Interestingly, PIKE-L knockdown elevated lactate-mediated autophagy as seen by increases in the autophagy biomarker microtubule-associated protein 1A/1B-light chain 3 (LC3-II), whereas autophagy was suppressed by PIKE-L overexpression. (Fig. 2*E*, *Bottom*). Cellular ADP/ATP ratios corresponded with p-AMPK levels (Fig. 2*F*) and an LDH cell-death analysis (Fig. 2*G*). Accordingly, these findings suggest that AMPK activation mediates neurodegeneration induced by α-synuclein overexpression and that PIKE-L plays a crucial protective role by blocking AMPK activation.

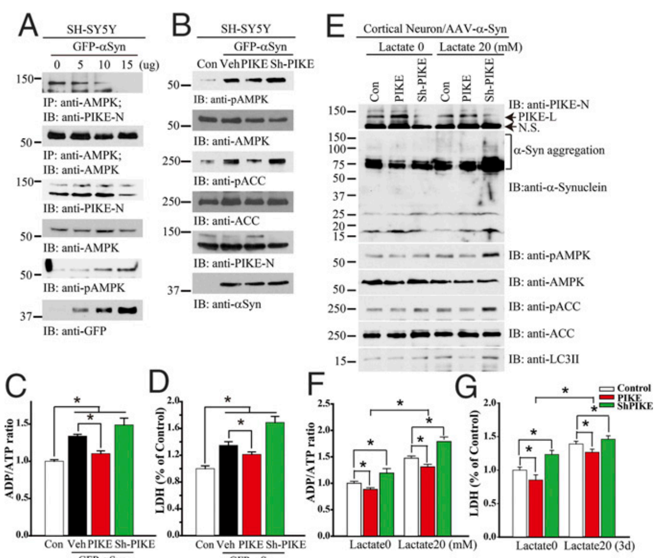

Fig. 2. PIKE suppresses AMPK phosphorylation and cell death induced by α-synuclein. (*A*) α-Synuclein competes with AMPK for binding to PIKE-L. GFP–α-synuclein was transfected into SH-SY5Y cells at different doses (0, 5, 10, and 15 μg). The binding of AMPK and PIKE-L was examined by immunoprecipitation. (*B*) PIKE mediates AMPK signaling activation induced by α-synuclein. GFP–α-synuclein was transfected into SH-SY5Y cells. Twenty-four hours later, the cells were infected with virus overexpressing PIKE, virus expressing PIKE shRNA, or vehicle. Forty-eight hours later, phosphorylation of AMPK and ACC was confirmed by immunoblotting. (*C*) Quantitative analysis of the ADP/ATP ratio in these cell lysates and cell media. (*D*) LDH analysis in these cell lysates and cell media. (*E*) Immunoblotting analysis of lactate-treated cells infected with PIKE-L or its shRNA. Cortical neurons were cultured and were infected with AAV–α-synuclein at 7 d in vitro and 2 d later were infected with Adeno-PIKE or Adeno–Sh-PIKE virus. On the day after Adeno virus infection, cells were treated with lactate (0 or 20 mM) for 3 d. α-Synuclein, p-S129, p-AMPK/AMPK, PIKE-L, and LC3-II were examined by immunoblotting. N.S., nonspecific band. (*F*) ADP/ATP ratios were examined in these lysates and cell media. (*G*) LDH analysis in these lysates and cell media. Data are shown as mean ± SEM ($n = 3$ each group). $^*P < 0.05$.

PIKE-L Is Required for DAergic Neuronal Survival in the MPTP Mouse Model. To investigate the physiological role of the PIKE-L/α-synuclein interaction in the MPTP model of PD, we treated wild-type mice, PIKE-KO mice, and Fyn-KO mice with saline or 15 mg/kg of MPTP two times/wk for 5 wk, followed by behavioral assays to assess motor functions. Tyrosine hydroxylase-positive (TH^+) neurons and terminals were reduced in all MPTP-treated substantia nigra and striatum (Fig. 3*A*). Quantitative immunohistochemistry using an LC3-II antibody revealed that MPTP triggered more robust autophagy in PIKE-KO and Fyn-KO mice than in wild-type mice (Fig. 3*B*). Notably, S129 phosphorylation was elevated upon MPTP treatment in all mice, and MPTP treatment increased the degree to which p-S129 α-synuclein bound to PIKE-L (Fig. 3*C*, *Top* and *Second*). As expected, AMPK and ACC phosphorylation was enhanced by MPTP because knockout of PIKE or Fyn activates the AMPK/ACC pathway (28, 29). Noticeably, MPTP treatment further elevated AMPK/ACC signal pathways (Fig. 3*C*, *Third* through *Sixth*). Moreover, immunoblotting of LC3-II was consistent with the quantitative data in Fig. 3*B*. Motor behavioral tests using the grid performance and the rotarod test demonstrated that MPTP treatment resulted in significant impairments in locomotor functions compared with saline (control) treatment. Remarkably, and in agreement with our histological findings, both MPTP-treated PIKE-KO and Fyn-KO mice exhibited much more severe motor impairments than wild-type mice (Fig. 3 *D* and *E*). MPTP induced much stronger reductions of both DA and DOPAC in PIKE-null and Fyn-KO mice than in wild-type mice (Fig. S4 *A* and *B*). Quantitative densitometric analyses of TH immunoreactivity in both the substantia nigra and striatum supported the finding that MPTP produced greater DAergic neurodegeneration in PIKE-null and Fyn-KO mice than in wild-type mice (Fig. S4 *C* and *D*). In alignment with the hyperactivation of p-AMPK/p-ACC, the measured ADP/ATP ratio demonstrated that MPTP provoked stronger activity in both PIKE-null and Fyn-KO mice than in wild-type mice (Fig. S4*E*). Together, these data suggest that PIKE prevents MPTP-induced DAergic neurodegeneration and that Fyn negatively regulates the association between PIKE-L and α-synuclein. Therefore, depletion of Fyn stimulates α-synuclein to block the interaction between PIKE-L and AMPK, resulting in AMPK hyperactivation and the subsequent DAergic neurodegeneration.

PIKE-L Is Required for DAergic Neuronal Survival in the α-Synuclein Transgenic Mouse Model. To interrogate further the pathological role of PIKE in PD pathogenesis, we used adeno-associated virus (AAV) to overexpress α-synuclein unilaterally in the substantia nigra, an established model of nigrostriatal neurodegeneration. The contralateral (control) hemisphere was treated with AAV expressing GFP. Immunofluorescent analysis of TH immunoreactivity indicated that nigral α-synuclein overexpression resulted in significant nigrostriatal denervation in both PIKE-null and Fyn-KO mice compared with the GFP-treated contralateral hemisphere

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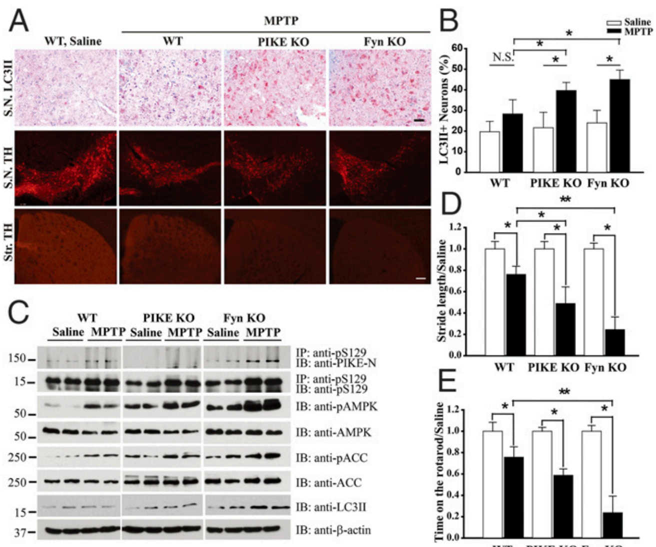

Fig. 3. MPTP induces AMPK hyperactivation in PIKE$^{-/-}$ and Fyn$^{-/-}$ mice, triggering DA neuron autophagy. Wild-type, PIKE-KO, or Fyn-KO mice were treated with MPTP two times/wk for 5 wk. (*A*, *Top Row*) DAergic neurons were greatly decreased by MPTP treatment in PIKE-KO or Fyn-KO substantia nigra. Autophagy in the damaged substantia nigra was measured via immunohistochemistry using LC3-II antibody. (Scale bar, 50 μm.) (*Middle* and *Bottom Rows*) Immunofluorescence with anti-TH shows the reduction of DAergic cell bodies in the substantia nigra and striatum of MPTP-injected wild-type, PIKE-KO, and Fyn-KO mice compared with saline-injected mice. (Scale bar, 200 μm.) (*B*) Quantification of autophagy. LC3-II$^+$ cells in the substantia nigra showed the increased neuronal autophagy in PIKE-KO and Fyn-KO mice compared with wild-type mice. (*C*) MPTP induces p-AMPK and LC3-II in PIKE-KO and Fyn-KO mice. The changes in protein levels in substantia nigra lysates from MPTP-treated animals were analyzed by immunoblotting. (*D* and *E*) Motor defects in mice were measured by performance in the grid (*D*) and rotarod (*E*) tests. Data are shown as mean ± SEM (n = 6–8 mice per group). $^*P < 0.05$, $^{**}P < 0.01$.

(Fig. 4*A*, *Middle* and *Bottom*). Moreover, autophagy (LC3-II staining) in the substantia nigra was increased in both PIKE-null and Fyn-KO mice (Fig. 4*A*, *Top*). Quantitative analysis showed that α-synuclein–induced LC3-II was significantly elevated in PIKE-null and Fyn-KO mice compared with wild-type mice. Again, the contralateral hemisphere treated with AAV-GFP displayed only a basal level of LC3-II, which was not different in any groups (Fig. 4*B*). Immunoblotting of substantia nigra lysates demonstrated that α-synuclein overexpression induced AMPK/ACC activation in wild-type mice. Notably, α-synuclein was clearly phosphorylated at S129 (Fig. 4*C*, *Left*). By contrast, α-synuclein overexpression in PIKE-null mice triggered a higher degree of AMPK/ACC phosphorylation and loss of TH. As expected, we found that S129 phosphorylation was significantly increased with the hyperactivation of AMPK in the absence of PIKE. Because Fyn blocks AMPK activation via both the liver kinase B1 (LKB1) and PIKE pathways (27–29), knockout of Fyn led to stronger activation of p-AMPK/p-ACC, which was further hyperactivated by α-synuclein. S129 phosphorylation was highly elevated in Fyn-KO mice (Fig. 4*C*, *Right*). Remarkably, autophagy (as measured by LC3-II levels) was enhanced by α-synuclein, and the levels of LC3-II showed an incremental increase from wild-type mice to PIKE-null mice and were highest in Fyn-null mice, tightly coupled to p-AMPK and p-S129 signals in all groups (Fig. 4*C*, *Right*). Behavioral analysis indicated that overexpression of α-synuclein significantly impaired motor functions in both PIKE-null and Fyn-KO mice compared with wild-type mice. Moreover, in comparison with GFP, α-synuclein overexpression triggered pronounced motor deficits in both PIKE-null and Fyn-KO mice but not in wild-type mice (Fig. 4 *D* and *E*). In alignment with DAergic neuronal loss, DA and its metabolite DOPAC were reduced in the substantia nigra and striatum of PIKE-null and Fyn-KO mice as compared with wild-type mice (Fig. S5 *A–D*). Quantitative analysis of TH immunoreactivity in the substantia nigra and striatum supported these findings (Fig. S5 *E* and *F*). Consistent with AMPK activation by α-synuclein overexpression, ADP/ATP ratios were greatly enhanced in wild-type mice and were further augmented in PIKE-null and Fyn KO mice (Fig. S5*G*). These findings suggest that hyperactivation of AMPK by α-synuclein overexpression in the absence of PIKE or Fyn leads to DAergic neuronal loss via autophagy and apoptosis.

AMPK Activation Mediates DAergic Cell Death Triggered by MPP$^+$. MPP$^+$ impairs mitochondrial respiration, resulting in the formation of high levels of reactive oxygen species that ultimately lead to apoptosis, necrosis, and autophagy, depending on the dose (Fig. S6) (30, 31).

AMPK Is Required for DAergic Neuronal Loss in the MPTP Mouse Model (Fig. S7). Detailed information is supplied in *SI Results*. Also see Figs. S6 and S7.

The AMPK Signaling Pathway Is Highly Activated in Dementia with Lewy Bodies. Our findings suggest that α-synuclein binds PIKE-L and sequesters it into Lewy bodies by stripping it away from AMPK, leading to hyperactivation of AMPK (and neurodegeneration in PD mouse models). To examine whether this hypothesis applies to human disease, we evaluated these signaling pathways in brains from patients with dementia with Lewy bodies (DLB) after ultracentrifugation. Compared with healthy control brains, PIKE-L

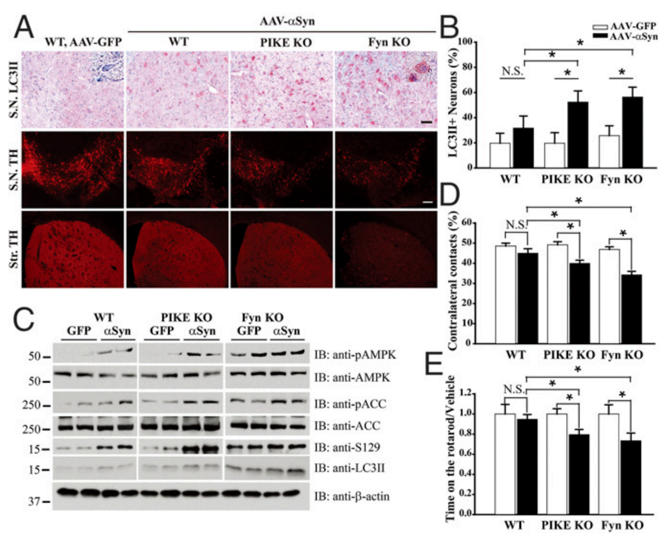

Fig. 4. α-Synuclein induces AMPK hyperactivation in PIKE$^{-/-}$ and Fyn$^{-/-}$ mice, triggering DA neuronal cell death via autophagy. AAV–α-synuclein or AAV-GFP was injected into the right substantia nigra of wild-type, PIKE-KO, and Fyn-KO mice. The animals were killed 2 mo after vector delivery. Overexpression of α-synuclein enhances autophagy in PIKE-KO or Fyn-KO mice. (*A*, *Top Row*) Autophagy in damaged substantia nigra was shown by immunohistochemistry using LC3-II antibody. (Scale bar, 50 μm.) (*Middle* and *Bottom Rows*) Immunofluorescence with anti-TH showed the reduction of DAergic cells in substantia nigra and nigrostriatal terminals in wild-type, PIKE-KO, and Fyn-KO mice injected with AAV–α-synuclein compared with mice injected with AAV-GFP. (Scale bar, 200 μm.) (*B*) Quantification of autophagy. An increase in LC3-II$^+$ cells in the substantia nigra demonstrates increased neuronal autophagy in PIKE-KO and Fyn-KO mice compared with wild-type mice. (*C*) α-Synuclein overexpression induces p-AMPK and autophagy in PIKE-KO and Fyn-KO mice. The changes in protein levels in lysates of substantia nigra with overexpressed α-synuclein were analyzed by immunoblotting. (*D* and *E*) Motor defects in mice were measured by the cylinder (*D*) and rotarod (*E*) tests. Data are shown as mean ± SEM (n = 6–8 mice per group). $^*P < 0.05$.

content in the supernatant was reduced in the brains from DLB patients compared with control brains; accordingly, its levels were increased in the pellet fraction of samples from DLB patients. On the other hand, α-synuclein monomer levels were reduced in the supernatant of DLB brain samples, but its aggregated/oligomeric form was elevated in the pellet (Fig. 5*A*, *First* to *Fourth*). Interestingly, the total level of Fyn was reduced in the DLB brains compared with brains from healthy controls. Consistently, p-AMPK/p-ACC and α-synuclein p-S129 were significantly enhanced in DLB brains compared with controls (Fig. 5*A*, *Fifth* through *Ninth*). As expected, levels of the autophagy marker LC3-II were markedly higher in DLB brains than in controls. Interestingly, the apoptotic marker active caspase-3 was slightly higher in DLB brains than in controls (Fig. 5*A*), indicating that neuronal cell death in synucleinopathies might be caused primarily by AMPK-mediated excessive autophagy combined with neuronal apoptosis. ADP/ATP ratios also supported the finding that AMPK was highly activated in DLB brains as compared with control brains (Fig. 5 *B* and *C*). PIKE-L and Fyn were significantly reduced in DLB brains compared with control brains (Fig. 5 *D* and *E*). Collectively, our in vitro, in vivo, and human patient data support the hypothesis that aggregated p-S129 α-synuclein binds strongly to PIKE-L and sequesters it into the Lewy bodies. This process, which is negatively regulated by Fyn tyrosine kinase, ultimately leads to AMPK hyperactivation and DAergic neurodegeneration.

Discussion

In the current report we show that α-synuclein overexpression or MPTP administration triggers AMPK/ACC signaling activation, leading to the phosphorylation of α-synuclein S129 by activated AMPK; that the α-synuclein/PIKE-L association is dependent on S129 phosphorylation; and that this action is mediated by AMPK-mediated phosphorylation of α-synuclein S129. This result is consistent with previous findings (32) in which McFarland et al. used proteomics/mass spectrometry to demonstrate that p-S129 synuclein selectively binds PIKE. Overexpression of PIKE-L represses the DAergic neurodegeneration induced by lactate or α-synuclein overexpression (Fig. 2). On the other hand, knockout of PIKE-L or Fyn leads to AMPK hyperactivation by MPTP or α-synuclein overexpression, triggering DAergic neuronal death (Figs. 2–5). Conceivably, p-S129 α-synuclein sequesters PIKE-L into the Lewy body inclusions. As expected, AMPK signaling is hyperactivated in PD brains, inducing extensive autophagy and some degree of apoptosis, as previously reported in human PD brain samples (33). Our findings described herein support the following molecular model: MPTP and α-synuclein interfere with complex I in the electron transport system in the mitochondria (34–36), causing mitochondrial dysfunction and the resultant oxidative stress and ultimately resulting in energy depletion (elevation of the ADP/ATP ratio) and AMPK activation. The activated AMPK phosphorylates α-synuclein on S129, elicits its association with PIKE-L, and sequesters it into Lewy bodies. This process subsequently removes PIKE-L's inhibitory effect on AMPK activation, results in a feed-forward scenario with increased S129 phosphorylation by AMPK, and increases the formation of the α-synuclein/PIKE-L complex. This process is further escalated by reduced levels of Fyn, which lead to LKB1 activation and concurrent AMPK activation (29). On the other hand, diminished Fyn tyrosine kinase disrupts the formation of the PIKE-L/α-synuclein complex. These interrelated processes ultimately induce AMPK hyperactivation, triggering neurodegeneration via excessive autophagy, accompanied by apoptosis in nigral neurons of PD brains (Fig. 5*F*). It is worth noting that we have reported previously that PIKE binds Akt and elevates its kinase activity and that knockout of PIKE diminishes its kinase activity (16, 24, 37). Sequestration of PIKE into the Lewy bodies in PD may strip PIKE away from both Akt and AMPK, additively contributing to neuronal cell death by inhibiting prosurvival signaling from Akt and amplifying the autophagic effects from hyperactive AMPK. Fyn inhibits AMPK activation via both the LKB1 pathway and the PIKE-L/α-synuclein complex; thus, depletion of Fyn exhibits stronger p-AMPK signals than PIKE knockout or

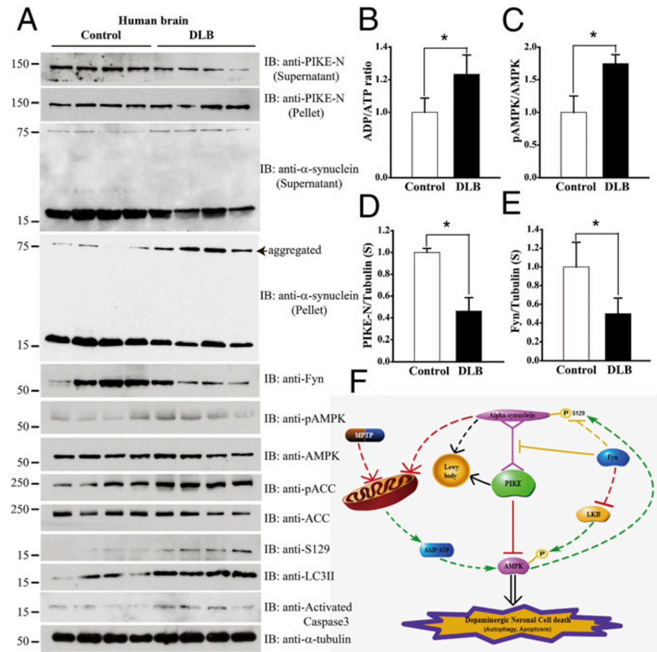

Fig. 5. AMPK is hyperactivated in patients with DLB and is associated with substantial autophagy in the substantia nigra. (*A*) PIKE-L is sequestrated by α-synuclein into insoluble inclusions in the brains of humans with DLB. The changes in PIKE-L, Fyn, and α-synuclein were examined by immunoblotting of samples from brains of healthy controls and patients with DLB. Phosphorylation of AMPK and ACC was confirmed. Moreover, autophagy and apoptosis were verified by immunoblotting of anti–LC3-II and anti-active caspase-3. (*B*–*E*) Quantitative analysis of ADP/ATP ratios (*B*), p-AMPK/AMPK (*C*), PIKE-L (*D*), and Fyn (*E*) in brains from controls and patients with DLB. The increase in the ADP/ATP ratio in patients with DLB was measured in human brain lysates (*B*). Densitometric analysis of immunoblotting shows the increase in p-AMPK (*C*) and the reduction of PIKE-L (*D*) and Fyn (*E*) in DLB tissues compared with controls. Data are shown as mean ± SEM ($n = 4$ each group). $*P < 0.05$. (*F*) The schematic model demonstrates that α-synuclein and MPTP might induce hyperactivation of AMPK and DAergic neuronal death by sequestering PIKE-L so that it cannot inhibit AMPK. Solid lines indicate results proved in the present study, and dashed lines indicate results from previous studies.

knockdown alone and results in more severe DAergic neuronal loss and motor deficits than seen in PIKE-null mice (Figs. 3–5). To discern which form of cell death is mainly responsible for MPP^+-induced AMPK activation in DAergic cells, we found that depletion of either of PIKE-L or Fyn activated AMPK, leading to robust autophagy and apoptosis, which could be blocked by dominant-negative AMPK (AMPK-DN). It was notable that the inhibition of autophagy in SH-SY5Y DAergic cells triggered by MPP^+ via 3-methyladenine (3-MA) gradually reduced cell death associated with LDH release (i.e., necrosis), whereas apoptosis increased progressively (Fig. S6). Hence, these findings suggest that AMPK might mediate DAergic cell death upon MPP^+ treatment primarily through excessive autophagy, in keeping with the controversial view of apoptosis in PD (38). Accordingly, in PIKE-null and Fyn-KO mice, overexpression of AMPK-DN suppressed MPTP-induced autophagy in the substantia nigra, leading to improved DAergic neuronal survival and motor function (Fig. S7). These findings are in agreement with a previous report that blockade of AMPK activation by the expression of AMPK-DN strongly inhibits DA neuron atrophy with moderate suppression of apoptosis and that overactivation of AMPK conversely strengthens DA neuronal degeneration induced by 6-hydroxydopamine (6-OHDA) (39). Furthermore, suppression of AMPK activity, either pharmacologically or genetically, exerts neuroprotective effects in cerebral ischemia (9, 40). Taken together, our results provide compelling evidence supporting the role of AMPK in DAergic cell death in

PD pathogenesis. Specifically, α-synuclein overexpression or treatment with neurotoxins elicits AMPK activation, which subsequently phosphorylates S129, triggering α-synuclein binding to PIKE-L and the sequestration of the latter into Lewy bodies. This action alleviates the inhibitory effect of PIKE-L on AMPK activation, leading to AMPK hyperactivation and DAergic cell loss via excessive autophagy in PD.

Methods

Animal care and handling were performed according to the Declaration of Helsinki and Emory Medical School guidelines. The protocol was reviewed and approved by the Emory Institutional Animal Care and Use Committee. Investigators were blinded to the group allocation during the animal experiments.

Human Tissue Samples. Postmortem brain samples were dissected from frozen brains from four control cases (age 71.2 ± 20.2 y, mean ± SEM) and four DLB cases (age 73.8 ± 9.3 y, mean ± SEM) from the Emory Alzheimer's Disease Research Center. The study was approved by the biospecimen committee of Emory University. DLB cases were clinically diagnosed and neuropathologically confirmed.

In Vitro Aggregation of α-Synuclein. Purified α-synuclein protein (rPeptide, 5 mg/mL) was first incubated with vehicle or the PH domain of PIKE for 1 h and was dialyzed against PBS, pH 7.0. The samples were incubated at 37 °C with continuous shaking for 3 d. The aggregation kinetics of α-synuclein was measured using Thioflavin T staining. The remaining solutions of aggregated α-synuclein were centrifuged at 100,000 × *g* for 30 min to separate the aggregated α-synuclein pellet and nonaggregated α-synuclein supernatant and were analyzed by Western blot.

MPTP Injection. MPTP (15 mg/kg) was injected s.c., and probenecid (250 mg/kg), a MPTP clearance inhibitor, was injected i.p. 10 times (every 3.5 d for 5 wk) to five groups: wild-type mice ($n = 7$), PIKE-KO mice ($n = 7$), Fyn-KO mice ($n = 8$), wild-type mice injected with AMPK-DN ($n = 8$), and Fyn-KO mice injected with AMPK-DN ($n = 8$). Control mice for each group ($n = 8$ in each group) received saline s.c. and probenecid MPTP i.p.

Cell Quantification. The number of TH^+ cells in the substantia nigra and striatum was estimated by ImageJ software using fluorescence intensity. For each animal, three consecutive sections of the substantia nigra and striatum were analyzed. For quantification of LC3-II^+ cells, the stained color was selected and set to the proper threshold for the binarization of the selected color image. The total number of immunoreactive neurons was analyzed using the same threshold (ImageJ). The investigator was blinded to the conditions of the analysis.

Statistical Analysis. Statistical analysis was performed using either Student's *t* test (for two-group comparisons) or one-way ANOVA followed by the least significant difference post hoc test (for comparisons of more than two groups). Differences with *P* values less than 0.05 were considered significant.

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