Delta-secretase Phosphorylation by SRPK2 Enhances its Enzymatic Activity, Provoking the Pathogenesis in Alzheimer’s Disease

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Summary

Delta-secretase, a lysosomal asparagine endopeptidase (AEP), simultaneously cleaves both APP and Tau, controlling the onset of pathogenesis of Alzheimer’s disease (AD). However, how this protease is post-translationally regulated remains unclear. Here we report that Serine-arginine protein kinase 2 (SRPK2) phosphorylates delta-secretase and enhances its enzymatic activity. SRPK2 phosphorylates serine 226 on delta-secretase and accelerates its autocatalytic cleavage, leading to its cytoplasmic translocation and escalated enzymatic activities. Delta-secretase is highly phosphorylated in human AD brains, tightly correlated with SRPK2 activity. Overexpression of phosphorylation mimetic (S226D) in young 3XTg mice strongly promoted APP and Tau fragmentation, and facilitated amyloid plaque deposits and neurofibrillary tangles (NFT)

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AUTHOR CONTRIBUTIONS
K.Y. conceived the project, designed the experiments, and wrote the manuscript. Z.-H.W. and P.L. designed and performed most of the experiments. X.L. prepared primary neurons, and assisted with in vivo and in vitro experiments. I.M.S. and F.M. prepared the delta-secretase viruses and critically read the manuscript. S.Y. performed electrophysiological experiments. J.-Z.W. designed the experiments, assisted with data analysis and interpretation and critically read the manuscript.
formation, resulting in cognitive impairment. Conversely, viral injection of the non-phosphorylatable mutant (S226A) into 5XFAD mice decreased APP and Tau proteolytic cleavage and attenuated AD pathologies and reversed cognitive defects. Our findings support that delta-secretase phosphorylation by SRPK2 plays a critical role in aggravating AD pathogenesis.

**Graphical abstract**

**Introduction**

Serine-Arginine protein kinase 2 (SRPK2) belongs to a small protein kinase family that specifically phosphorylates serine residues of the serine-arginine (SR) rich motif on substrates (Wang et al., 1999; Wang et al., 1998). It is a cell cycle-regulated kinase that modulates the activity of the pre-mRNA alternative splicing machinery (Ngo et al., 2005; Zhong et al., 2009; Zhou et al., 2012). A cell cycle signal induces nuclear translocation of SRPK2 at the G2/M boundary (Ding et al., 2006). Previously, we reported that Akt-mediated phosphorylation of SRPK2 at T492 controls the expression of cell cycle regulators, thus coupling cell cycle regulation and cell death machinery in the nervous system. Consequently, activation of SRPK2 in neurons leads to cell cycle progression and DNA synthesis and eventually apoptosis (Jang et al., 2009). Moreover, we showed that SRPK2 is cleaved by caspase 3 to induce apoptosis (Hong et al., 2011). Interestingly, SRPK2 can be activated by Aβ, and activated SRPK2 phosphorylates Tau on S214, suppressing Tau-dependent microtubule polymerization and inhibiting axonal elongation in neurons. SRPK2 activity is augmented in the neurons of Alzheimer’s disease (AD) mice and patients (Hong et al., 2012), strongly implicating a role for SRPK2 in AD disease etiology and pathogenesis. Our previous work also showed that SRPK2 phosphorylates SC35, a SR splicing factor located in nuclear speckles (Jang et al., 2009), and it has recently been reported that dysregulated RNA processing with accumulation of unspliced RNA species occurred in human AD and MCI (mild cognitive impairment) patients (Bai et al., 2013).
AD is characterized by the accumulation of β-amyloid peptide (Aβ) within the brain along with hyperphosphorylated and cleaved forms of the microtubule-associated protein Tau. The physiopathology of AD is not yet totally established. Nevertheless, it is known that a dysfunction in the metabolism of the amyloid β precursor protein (APP) leads to the formation of neuritic plaques and abnormal Tau phosphorylation results in neurofibrillary tangles (NFT) (Mattson, 2004). Mammalian asparaginyl endopeptidase (AEP, gene name LGMN) is a lysosomal cysteine protease that cleaves after asparagine residues. AEP is synthesized as a zymogen (pro-AEP, 56 kDa) and is autocatalytically processed into active AEP under acidic conditions (Li et al., 2003). We recently reported that AEP cleaves both APP and Tau in human AD brains. Interestingly, AEP expression level and activity are escalated in aged mice and AD brain (Zhang et al., 2014; Zhang et al., 2015). These findings indicate that AEP might act as a novel delta-secretase in AD progression. AEP (referred to as delta-secretase from hereon) cuts APP at both the N373 and N585 residues in the ectodomain and facilitates Aβ production by decreasing the steric hindrance for BACE1. Depletion of delta-secretase significantly reduces Aβ production and senile plaque formation in the 5XFAD mouse brain, leading to restoration of synaptic activity and cognitive function (Zhang et al., 2015). Additionally, delta-secretase also cleaves Tau at N255 and N368 and abolishes its microtubule assembly activity, resulting in its aggregation and NFT formation. Moreover, the cleaved Tau 1–368 fragment is neurotoxic. Deletion of delta-secretase from Tau P301 mice reverses the synaptic plasticity defect and cognitive dysfunction (Zhang et al., 2014). Accordingly, inhibition of delta-secretase using a small molecule inhibitor potently decreases amyloid plaques deposits and NFT in both 5XFAD and Tau P301S mouse models, improving cognitive function (Zhang et al., 2016; Zhang et al., 2017). However, how this critical protease is regulated post-translationally during ageing and AD pathogenesis remains elusive. In the current report, we show that SRPK2 selectively phosphorylates delta-secretase on Serine 226 and facilitates its activation, elevating its enzymatic activity in human AD brains. Expression of non-phosphorylatable mutant (S226A) in 5XFAD mice attenuates the AD pathology, rescuing cognitive function. Conversely, expression of the phosphorylation mimetic S226D in young 3XTg mice promotes an earlier onset of senile plaques deposits and NFT formation, accelerating cognitive dysfunction.

**Results**

**SRPK2 phosphorylates Serine 226 of delta-secretase**

Both SRPK2 and delta-secretase are activated in an age-dependent manner in AD mouse models and in human AD brains, triggering deleterious effects in this neurodegenerative disease. Moreover, both proteins regulate Tau pathological functions via either phosphorylation or cleavage (Hong et al., 2012; Zhang et al., 2014; Zhang et al., 2015). Accordingly, we hypothesize that these two pathways are linked in that SRPK2 might regulate delta-secretase via phosphorylation. To test this hypothesis, we conducted an in vitro kinase assay with purified His-tagged AEP recombinant protein in the presence of γ-32P-[ATP]. We observed that His-AEP recombinant protein was strongly phosphorylated by wild-type (WT) SRPK2 but not kinase-dead (KD) SRPK2 (Figure 1A). As a positive control, we utilized GST-Tau recombinant protein. To further explore whether SRPK2
phosphorylates delta-secretase in intact cells, we cotransfected mammalian GST-tagged (mGST) delta-secretase with WT or KD SRPK2 into HEK293 cells. Transfected delta-secretase was pulled down and examined by immunoblotting with anti-p-S/T antibody. We found that GST-delta-secretase associated with WT SRPK2 and that both proteins in this complex were phosphorylated (Figure 1B). Delta-secretase activation requires sequential removal of its N- and C-terminal peptides at residues D25 and N323 (Li et al., 2003). Accordingly, we assessed the extent of phosphorylation of the mature version of delta-secretase by SRPK2. We found that the delta-secretase a.a. 26–323 was phosphorylated to a higher degree than full-length delta-secretase (Figure 1C), indicating that the phosphorylation sites are located within this mature domain. Since SRPK2 predominantly phosphorylates the substrate at a SR dipeptide (Wang et al., 1998), we identified two domains (a.a. 225–226 and a.a. 328–329; Figure 1D, top panel) within delta-secretase. To test the ability of SRPK2 to phosphorylate these motifs, we generated point mutants (S226A, S328A) and dual mutant (S226A/S328A) and performed in vitro kinase assay. We found that both the S226A and S328 mutations evidently reduced delta-secretase phosphorylation and the removing both motifs largely eliminated phosphorylation (Figure 1D, middle panel). Moreover, GST pulldown assay in intact cells demonstrated that S226A mutation completely abolished delta-secretase phosphorylation by SRPK2, whereas the S328 mutation did so to a much lesser extent. This suggests that S226 might be the major SRPK2 phosphorylation residue on delta-secretase (Figure 1E, top panel). To determine whether delta-secretase is a physiological substrate of SRPK2, we generated a phospho-S226-specific antibody. Indeed, delta-secretase S226 phosphorylation tightly correlated with our anti-phospho-S/T antibody results (Figure 1E, 2nd panel). To validate the specificity of anti-p-S226 antibody, we cotransfected WT or the non-phosphorylatable S226A mutant into HEK293 cells with WT or KD SRPK2. Wild-type delta-secretase was selectively phosphorylated by WT, but not KD, SRPK2, which was visualized by anti-p-S226 antibody. Importantly, p-delta-secretase signals were completely abolished by the co-incubation with the phosphopeptide antigen, demonstrating the specificity of detection of this phosphorylation (Figure S1A). As expected, p-S226 antibody specifically recognized phosphorylated delta-secretase in control but not in delta-secretase-depleted human SY5Y cells. Noticeably, the truncated mature form was more strongly phosphorylated than full-length delta-secretase (Figure S1B). Thus, these results suggest that S226 is a major SRPK2 phosphorylation target on delta-secretase.

**SRPK2 phosphorylates delta-secretase in human AD brains**

Our previous study shows that SRPK2 is activated and phosphorylated in AD brains (Hong et al., 2012). To assess whether Aβ activates SRPK2 to phosphorylate AEP in primary neurons, we performed immunofluorescent (IF) staining to find that Aβ induced SRPK2 T492 and deltasecretase S226 phosphorylation in a dose-dependent way (Figure 2A & B), and Aβ treatment triggered the subcellular co-localization of these two proteins as well (Figure 2C & D). To test whether SRPK2 is indeed implicated in phosphorylating delta-secretase in neurons, we knocked down SRPK2 with lentivirus-mediated expression of shRNA, followed by Aβ treatment overnight. We found that Aβ triggered SRPK2 T492 phosphorylation, which is a marker for its activation (Jang et al., 2009). Knockdown of SRPK2 eliminated T492 phosphorylation by Aβ. As expected, incubation with Aβ also resulted in delta-secretase fragmentation and S226 phosphorylation, which was inhibited.
with SRPK2 knockdown (Figure 2E, top to 4th panel). As a result, we saw an increase in the cleavage of downstream delta-secretase targets, including APP and Tau (APP N585 and Tau N368). The extent of cleavage was mitigated with SRPK2 knockdown (Figure 2E, 5th to bottom panels). This suggests that Aβ triggers SRPK2 activation in primary neurons, which subsequently phosphorylates delta-secretase, inducing its proteolytic activity. We virtually made the same observations in human SY5Y cells (Figure S2A). Next, we extended our studies into human AD brains, where we found a high level of S226 phosphorylation of delta-secretase (associated with its active fragments). In contrast, deltasecretase was barely cleaved or phosphorylated in healthy controls brains. Delta-secretase S226 phosphorylation correlated well with SRPK2 phosphorylation, as well as with the phosphorylation of SRPK2 substrates such as p-Tau S214 and p-Acinus S422 (Jang et al., 2008) (Figure 2F). Immunofluorescence staining with anti-phosphor-/total delta-secretase and SRPK2 showed prominent colocalization between SRPK2 and delta-secretase, and increased expression of SRPK2 pT492, delta-secretase and delta-secretase pS226 in human AD hippocampal CA1 than healthy controls (Figure S2B-C). Together, these results suggest that delta-secretase S226 is phosphorylated by SRPK2 in human AD brains.

AEP (delta-secretase) is a lysosomal cysteine protease which is activated in the AD brain where it is translocated from neuronal lysosomes to the cytoplasm (Basurto-Islas et al., 2013). To determine whether S226 phosphorylation is involved in this process, we conducted a subcellular fractionation. Interestingly, we found that mature and active delta-secretase in AD patients were more abundant than healthy controls in both cytosolic and lysosomal fractions, and the active truncate was highly phosphorylated on S226 in AD (Figure 2G). To further test whether S226 phosphorylation dictates delta-secretase cytoplasm translocation from the lysosomes, we transfected HEK293 cells with GST-tagged WT, S226A, or S226D delta-secretase, and performed co-staining with FITC-conjugated anti-GST antibody and LAMP1, a specific marker for lysosomes. Interestingly, we found that the phosphorylation mimetic mutant S226D was localized to the cytoplasm, whereas both WT and S226A predominantly co-localized with anti-LAMP1, indicating that WT and non-phosphorylatable delta-secretase mainly resides in the lysosomes, whereas phosphorylated delta-secretase translocates into the cytoplasm (Figure 3A & C). IF staining with anti-p-S226 in primary neurons showed that the Aβ-mediated augmentation of S226 phosphorylation (e.g. Figure 2B) correlated with increased cytosolic localization (Figure 3B & D). To further explore whether delta-secretase cytoplasmic translocation is associated with its p-S226 activity, we conducted a subcellular fractionation with Aβ peptide-treated SY5Y cells. Notably, Aβ strongly escalated delta-secretase S226 phosphorylation and cytosolic translocation. Again, the mature form in the cytosolic fraction was more pronouncedly phosphorylated than those in the lysosomes (Figure 3E). To further interrogate where and when SRPK2 might phosphorylate AEP, we transfected SY5Y cells with siRNA against SRPK2, followed by Aβ (10 µM) treatment for different time points. Subcellular fractionation showed that Aβ treatment elicited SRPK2 to phosphorylate delta-secretase in the lysosomes, subsequently, the phosphorylated and truncated (mature form) delta-secretase translocated into the cytosol. Moreover, Aβ incurred potent AEP S226 and SRPK2 T492 phosphorylation at 8 h in both the lysosome and the cytoplasmic fractions and it augmented in a time-dependent manner. Depletion of SRPK2 substantially abolished Aβ-induced delta-
Delta-secretase S226 phosphorylation enhances its autocatalytic process and enzymatic activity

To assess the biological effect of S226 phosphorylation on delta-secretase enzymatic activity, we cotransfected mGST-SRPK2 (WT or KD) and Myc-delta-secretase into HEK293 cells, and conducted delta-secretase enzymatic assay. We found that SRPK2 overexpression increased delta-secretase enzymatic activity as compared to control. By contrast, expression of KD SRPK2 decreased delta-secretase enzymatic activity (Figure 4A). Next, we analyzed endogenous delta-secretase enzymatic activity in the presence of SRPK2. Expectedly, compared to KD and vector control, WT SRPK2 expression also enhanced endogenous delta-secretase activity (Figure 4B). We also extended our studies into the delta-secretase S226D and S226A mutants, and found that the phosphorylation mimetic S226D displayed higher enzymatic activity than wild-type, and S226A mutant possessed lower enzymatic activity compared with wild-type (Figure 4C). Next, we measured delta-secretase autocatalytic fragmentation following different time points of incubation at 37°C. Immunoblotting revealed that delta-secretase proteolytic processing rates were the same as its enzymatic efficacy where S226D > wild-type > S226A (Figure 4D). To further test the effect of SRPK2 phosphorylation on delta-secretase proteolytic activity, we cotransfected Myc-SRPK2 (WT or KD) with mGST-delta-secretase in HEK293 cells, and incubated the cell lysates with GST-Tau for different time points. Delta-secretase was phosphorylated by WT but not KD SRPK2, fitting with its profile of enzymatic activity (Figure S4A, right upper panels). Immunoblotting showed that phosphorylated delta-secretase cleaved Tau in a time-dependent fashion, whereas non-phosphorylatable delta-secretase exhibited an extremely low degree of Tau cleavage (Figure S4A, left panels). Quantitative analysis of delta-secretase phosphorylation is shown in the right bottom panel of Figure S4A. We made
similar observations with Tau proteolytic degradation by delta-secretase S226D and S226A, which aligned with these mutants’ enzymatic activities (Figure 4E).

Our data support that p-S226 on delta-secretase facilitates its auto-processing into the mature form. To directly test this notion in vitro, we purified mGST-AEP WT and S226A mutant recombinant proteins, and performed in vitro phosphorylation with isolated Myc-SRPK2 WT or KD at pH 7.4 buffer for 1 h, then we initiated autocleavage at pH 6.0 for different time points. We found that SRPK2 phosphorylation elicited time-dependent AEP autocleavage, which was substantially reduced with S226A or KD mutant. Remarkably, the slowest auto-processing occurred to S226A + KD group, indicating AEP phosphorylation on S226 plays a key role in dictating AEP auto-cleavage and maturation (Figure S4B, upper panels). Moreover, we analyzed the in vitro phosphorylated delta-secretase recombinant protein’s enzymatic activity using well-characterized mGST-Tau as a substrate. Again, we found Tau fragmentation displayed the similar cleavage pattern to delta-secretase auto-processing (Figure S4B-C, lower panels). As expected, employing the mature and truncated delta-secretase (a.a 26–323) fragment and its S226A mutant, we found that stronger enzymatic activity with mature delta-secretase. Consequently, we monitored Tau fragmentation with shorter period of incubation (from full-length 30–60 to truncate 15–30 min), and discovered even more robust Tau fragment (Figure S4D-E), supporting that in vitro phosphorylation of delta-secretase truncate enhances its protease activity. Together, these data strongly support that SRPK2 phosphorylates delta-secretase and triggers its autocatalytic activation.

**Delta-secretase S226D facilitates AD pathologies in young 3XTg mice, stimulating cognitive dysfunctions**

A triple-transgenic mouse model (3XTg-AD) harboring PS1(M146V), APP(Swe), and tau (P301L) transgenes has been developed to study the interaction between Aβ and Tau, and their effect on synaptic functions. 3XTg-AD mice progressively develop amyloid plaques in hippocampus at 6 months old of age and NFT at 12 months (Oddo et al., 2003). To investigate whether expression of phosphorylated delta-secretase may facilitate the early onset of AD pathology, we injected the hippocampus of 2 month old wild-type mice and age-matched 3XTg mice with lentivirus expressing control, wild-type delta-secretase, or the S226D mutant (Figure S5A). Three months following the vector delivery, animals were sacrificed and analyzed for delta-secretase activity. Immunoblotting analysis revealed that animals overexpressing WT or S226D delta-secretase displayed mature active fragment, and this cleavage was observed at a higher degree with S226D overexpression as opposed to WT. Moreover, the level of active delta-secretase truncates was higher in 3XTg mice than in wild-type mice (Figure 5A, top panel). Accordingly, we also found the corresponding increase of Tau N368 and APP N585 fragments with the pattern of S226D > WT > control, fitting with the levels of active delta-secretase (Figure 5A, 2nd to bottom panels). An enzymatic assay showed the same pattern of activity, and 3XTg mice exhibited higher delta-secretase activity than wild-type mice (Figure 5B). Thioflavin S staining and IHC staining with an anti-Aβ showed that S226D overexpression resulted in more amyloid plaques deposits as compared to WT treatment. Very few plaques were found in control vector treated animals in the same hippocampal region (Figure 5C; Figure S5B). Accordingly, Aβ ELISA analysis
demonstrated that S226D overexpression produced significantly more Aβ peptide than that of wild-type delta-secretase, followed by control virus in 3XTg mice. However, Aβ levels remained comparable in wild-type mice regardless of delta-secretase wild-type or S226D expression (Figure S5C & D). Interestingly, AT-8 and AT-100 staining revealed that S226D overexpression induced Tau phosphorylation in the hippocampus as compared to wild-type and control treated animals (Figure 5D), which normally only occurs at 12 months of age or later (Oddo et al., 2003). Synaptic loss is believed to be the basis of cognitive impairment in the early phase of AD (Shankar and Walsh, 2009). To assess the physiological role of p-S226 on delta-secretase in this process, we performed electronic microscope (EM) analysis, which demonstrated that the synapses were gradually decreased, when WT or S226D delta-secretase was overexpressed in 3XTg mice. Notably, 3XTg/S226D mice possessed significantly fewer synapses than 3XTg/control or wild-type/S226D mice. By contrast, no change in synapse number was observed in wild-type mice, regardless of WT or S226D (Figure S5E). Dendritic spine density of hippocampal neurons corresponded to EM analysis (Figure 5E). Electrophysiological measurements demonstrated that long-term potentiation (LTP) was pronouncedly impaired in 3XTg mice than in wild-type mice. Again, S226D overexpression substantially reduced LTP in 3XTg as compared with control or WT delta-secretase groups (Figure 5F; Figure S5F). Next, we performed Morris Water Maze (MWM) behavioral test to evaluate the learning and memory capability. During the training phase, 3XTg mice displayed much longer latency to reach the platform than wild-type mice, and delta-secretase overexpression exacerbated this phenotype, where 3XTg/S226D mice performed the worst, followed by 3XTg/wild-type and 3XTg/control. A probe trial found that S226D overexpression significantly impaired memory in 3XTg mice as compared to WT delta-secretase or control virus treated mice (Figure 5G-H; Figure S5G). The swim speed was similar among the groups, indicating that delta-secretase expression does not affect motor function (Figure S5H). A cued fear conditioning assay showed that 3XTg/S226D mice exhibited less freezing as compared to 3XTg/control and 3XTg/wild-type (Figure S5I). Together, these results strongly support that SRPK2 phosphorylation of delta-secretase stimulates its activation and enzymatic activity, leading to early onset of AD pathology and acceleration of cognitive dysfunction in young 3XTg AD mice.

**Delta-secretase S226A reduces AD pathologies in 5XFAD mice, alleviating cognitive impairments**

Delta-secretase activity is elevated in 5XFAD mouse model and in human AD brains (Basurto-Islas et al., 2013; Zhang et al., 2015). To examine the pathological role of S226 phosphorylation in AD pathogenesis, we injected lentivirus expressing WT delta-secretase or the S226A mutant into the hippocampus of 2 months old mice (Figure S6A). Three months after the viral injection, we measured delta-secretase expression and activity by immunoblotting. In 5XFAD mice, overexpression of WT delta-secretase resulted in a higher level of formation of the active mature form of delta-secretase than S226A overexpression. Consistent with these findings, the level of both Tau N368 and APP N585 fragments were increased in WT delta-secretase overexpressing animals as compared to controls. In contrast, S226A overexpression reduced both Tau and APP proteolytic cleavage by delta-secretase. On the other hand, overexpression of wild-type delta-secretase, but not S226A, in wild-type mice resulted in the formation of active mature delta-secretase; however, to a lesser extent.
than that seen in 5XFAD mice. Consequently, Tau N368 and APP N585 were increased in wild-type delta-secretase treated animals as compared with control and S226A expressing mice (Figure 6A). Enzymatic activity was significantly increased in WT delta-secretase overexpressing brains, and no increase in enzymatic activity was seen with S226A expression. In line with the delta-secretase cleavage, enzymatic activity was higher in 5XFAD mice than in wild-type (Figure 6B). Aβ concentrations tightly correlate with APP N585 levels in the hippocampus of 5XFAD mice. However, no significant increase in Aβ peptides was seen wild-type mice (Figure S6B & C). Thioflavin S staining and IHC staining with anti-Aβ showed that amyloid plaque deposits was enhanced in WT delta-secretase injected hippocampi as compared to control samples. S226A mutant overexpression resulted in decreased amyloid plaque formation in 5XFAD mice (Figure 6 C & D). EM analysis revealed that delta-secretase overexpression substantially reduced synapse numbers in the hippocampus as compared to control. In contrast, S226A mutant overexpression increased the number of synapses (Figure S6D). These observations were confirmed via quantification of dendritic spines where we found that S226A mutant expression greatly increased spine density as compared to control, whereas overexpression of wild-type delta-secretase resulted in decreased spine density (Figure 6E). 5XFAD mice exhibit impaired LTP at Schaffer collateral-CA1 pathways (Kimura and Ohno, 2009). Delta-secretase overexpression reduced LTP as compared to control, whereas S226A expression rescued LTP. The paired-pulse ratios were lower in 5XFAD/wild-type as compared to 5XFAD/control, and 5XFAD/S226A subjects reversed the deficit, suggesting that inactivation of delta-secretase rescues the synaptic dysfunctions (Figure 6F). Next, we evaluated spatial memory in 5-month-old 5XFAD, 5XFAD/AEP and 5XFAD/S226A mice using the MWM. Compared to control, overexpression of delta-secretase wild-type significantly increased the latency to platform, whereas S226A reduced it (Figure 6G). On the probe trial, S226A group spent more time in the target quadrant than control, indicating better memory. By contrast, wild-type AEP expression spent less time in the quadrant (Figure 6H). All groups of mice exhibited comparable swim speed (Figure S6E). Similarly, cued fear conditioning test also demonstrated that S226A expression can rescue the cognitive defects in 5XFAD mice (Figure 6I). Together, these data support that expression of a non-phosphorylatable delta-secretase mutant ameliorates AD pathology in 5XFAD mice, rescuing the cognitive dysfunction associated with this model.

Discussion

In the present study, we show that SRPK2 phosphorylates delta-secretase on residue S226 and accelerates its autocatalytic process, resulting in enhanced delta-secretase enzymatic activity. The S226 phosphorylation enables delta-secretase to proteolytically cleave its substrates more efficiently, which in turn facilitates AD pathogenesis including amyloid plaque deposits and NFT formation. As a result, this phosphorylation substantially exacerbates synaptic loss, which leads to LTP impairment and cognitive dysfunction. On the other hand, inhibition of this phosphorylation by expression of the non-phosphorylatable mutant S226A reverses these biological and pathological actions, rescuing the cognitive deficits in AD mouse models. It is worth noting that overexpressing wild-type delta-secretase or the phosphomimetic S226D induces both Tau and APP proteolytic cleavage in
wild-type mice, however, we failed to observe any prominent pathological or electrophysiological effects in these mice. Presumably, the truncated Tau N368 and APP N585 produced by delta-secretase are efficiently cleared from wild-type brains, whereas there are much more abundant APP and Tau substrates in 3XTg mice. Thus, the production rate of APP N585 (and subsequent Aβ peptides) and Tau N368 is higher than the rate of clearance, leading to senile plaques and NFT accumulation in 3XTg mice.

Accumulating evidence demonstrates that delta-secretase (AEP) plays a critical role in a variety of human diseases including cancers and neurodegenerative diseases (Basurto-Islas et al., 2013; Lin et al., 2014; Liu et al., 2003; Ohno et al., 2013; Zhang et al., 2016). However, how this seemingly deleterious protease is post-translationally regulated remains incompletely understood. Previous studies reveal delta-secretase modification like glycosylation (Chen et al., 1997) and ubiquitination by TRAF6 (Lin et al., 2014). This is however, the first study investigating the role of delta-secretase phosphorylation in protein function and disease. Delta-secretase is synthesized as a zymogen (pro-AEP, 56 kDa) and autocatalytically processed into active delta-secretase under acidic conditions. Processing of delta-secretase requires sequential removal of the C- and N-terminal propeptides at different pH thresholds to generate 46-kDa (active pro-AEP) and 36-kDa (active AEP) active enzymes (Halfon et al., 1998; Li et al., 2003). It remains unknown whether glycosylation or ubiquitination plays any role in this activation process. However, in the current study, we provide extensive evidence demonstrating that Aβ-activated SRPK2 selectively phosphorylates delta-secretase on S226, within a SR dipeptide motif, and accelerates its autocatalytic process. Importantly, we found that delta-secretase is highly phosphorylated in human AD brains, tightly coupled to the activation of its upstream kinase SRPK2 (Figure 2). Moreover, since delta-secretase is greatly upregulated in numerous human cancers (Gawenda et al., 2007; Li et al., 2003; Zhu et al., 2016), one may postulate that active delta-secretase may also be robustly phosphorylated on S226 by SRPK2, mediating cancer cell cycle progression, cell proliferation, and invasion (Andrade et al., 2011).

Terminally differentiated neurons are unable to re-enter the cell cycle, but accumulating evidence has demonstrated the up-regulation of cell cycle regulatory proteins in degenerating neurons of AD brains (Husseman et al., 2000). Moreover, SRPK2 is regulated by the cell-cycle. Remarkably, we also found that SRPK2 T492 phosphorylation escalated during the cell cycle progression, correlating with delta-secretase S226 phosphorylation that mediated delta-secretase cytoplasmic translocation from the lysosome (Figure 3). Our earlier study showed that SRPK2 translocates from the cytoplasm into the nucleus and phosphorylates SC35, a SR domain containing splicing factor, triggering cell cycle transition in neurons, thus inducing apoptosis through regulation of nuclear cyclin D1. Interestingly, SRPK2 is phosphorylated on T492 by activated Akt in the ischemia-damaged brain (Jang et al., 2009). Moreover, we have also reported that SRPK2 binds and phosphorylates acinus, an SR protein essential for RNA splicing and apoptosis (Sahara et al., 1999; Schwerk et al., 2003), and redistributes it from the nuclear speckles to the nucleoplasm, resulting in cyclin A1, but not A2, up-regulation. Hence, SRPK2 phosphorylates the SR splicing machinery and regulates cell cycle and contributes to leukemia tumorigenesis.
Recently, we reported that SRPK2 phosphorylates Tau and suppresses Tau-dependent microtubule polymerization, inhibiting axonal elongation in neurons. Depletion of SRPK2 in dentate gyrus inhibits Tau phosphorylation in APP/PS1 mouse and rescues LTP, alleviating cognitive impairment. Moreover, active SRPK2 is increased in APP/PS1 mice cortex and in the human AD brain (Hong et al., 2012). In AD, cell cycle defects and associated aneuploidy have been described. The aneuploid and hyperploid neurons that arise in AD are particularly prone to degeneration and could account for 90% of the neuronal loss that characterizes late-stage AD (Arendt et al., 2010). Individuals with Tau P301L mutation, which is associated with frontotemporal dementia, have several chromosome aberrations, such as aneuploidies in their fibroblasts and lymphocytes (Rossi et al., 2008). Consequently, increased level of aneuploidy in splenic lymphocytes of tauopathy transgenic mouse models have recently been reported (Rossi et al., 2014). In Drosophila, human Tau excess impairs mitosis, leading to aneuploidy and cell death (Bouge and Parmentier, 2016). Plausibly, abnormal activation of SRPK2 may phosphorylate numerous substrates including delta-secretase, Tau, SR domain containing splicing factors, coordinating the AD pathologies, coupling defective cell cycle machinery to neuronal cell death. Selective blockade of SRPK2 or delta-secretase by small molecular inhibitor may provide an innovative therapeutic treatment for the devastating neurodegenerative disease.

STAR METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for reagents may be directed to, and will be fulfilled by the Lead Contact, Keqiang Ye (kye@emory.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice, primary cultured rat neurons, cell lines, and human tissue samples—Wild-type C57BL/6J mice, 3xTg and 5XFAD mice were ordered from the Jackson Laboratory (000664, 34830, 34840). Animal care and handling was performed according to NIH animal care guidelines and Emory Medical School guidelines. The protocol was reviewed and approved by the Emory Institutional Animal Care and Use Committee. Primary rat cortical neurons were cultured as previously described (Zhang et al., 2014). All rats were purchased from the Jackson Laboratory. The protocol was reviewed and approved by the Emory Institutional Animal Care and Use Committee. HEK293 and HeLa cells were cultured in high-glucose DMEM added with 10% fetal bovine serum (FBS) and penicillin (100 units/ml) - streptomycin (100 µg/ml) (all from HyClone). SH-SY5Y cells were cultured in DMEM/F12 added with 10% FBS and penicillin (100 units/ml) - streptomycin (100 µg/ml) (all from HyClone). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO2. Post-mortem brain samples were dissected from frozen brains of AD and aged-match non-demented controls from the Emory Alzheimer’s Disease Research Center. The study was approved by the Biospecimen Committee. AD was diagnosed according to the criteria of the Consortium to Establish a Registry for AD and the National Institute on Aging. Diagnoses were confirmed by the presence of amyloid plaques and neurofibrillary tangles in formalin-fixed tissue. Informed consent was obtained from the subjects.
METHOD DETAILS

Transfection and infection of the cells—The coding sequence of human SRPK2 was inserted into pFUGW between Xba I and Mfe I or into pEGX-4T-2 between Sal I and Not I. The shRNA sequences targeting SRPK2 were fused into the lentiviral vector pFH1UGW. The target sequences recognized by shSRPK2 (5′- GCAGAGAGTGATTACACGTAT-3′) were acquired from Sigma-Aldrich MISSION shRNA library. HEK293 transfection was performed using Lipofectamine 2000 (Invitrogen). SH-SY5Y transfection was performed using Lipofectamine 3000 (Invitrogen). The lentiviruses used in neuron infection were packaged in VVC of Emory University. Transfection of the lentiviral vectors were performed in HEK293FT cells (50%-60% confluent). After 16 h, the transfection media were discarded, and the cells were washed with sterile PBS and incubated with pre-warmed fresh culture media. After 48 h incubation, the cell culture media were collected, and the lentiviral particles were concentrated by ultracentrifugation at 22,000 RPM (SW28) for 2 h at 4 °C. All the lentivirus-containing materials were handled according to the Biosafety regulation of Emory University. AEP lentiviruses WT/S226A/S226D used in vivo were packaged by the Manfredsson laboratory at Michigan State University as previously described (Benskey, M.J. and Manfredsson, F.P., 2016). Viral Titer is around 1–5 × 10^9 IU/ml.

In Vitro Kinase Assay—Human recombinant SRPK2 and Tau proteins were expressed in HEK293 cells and purified using Glutathione Sepharose™ 4B Beads or by immunoprecipitation. Recombinant human AEP proteins was bought from Novoprotein (C-371). Purified SRPK2 proteins were incubated with AEP proteins or Tau proteins in kinase reaction buffer (25 mM Tris, pH 7.5, with 10 mM MgCl2, 2 mM DTT, 5 mM β-Glycerolphosphate, 0.1 mM Na3VO4, and 2 mM EGTA) containing 20 µM ATP and 1 µCi of [γ-32P]-ATP for 30 min at 30 °C. Reactions were then separated in SDS-PAGE and analyzed by autoradiography.

Subcellular Fractionation—The protocol was as previously described (Basurto-Islas et al., 2013). Brain tissue and SH-SY5Y cell lysates were prepared in 3 volumes of cold homogenizing buffer (0.32 M sucrose, 1 mM EDTA disodium, 10 mM HEPES, pH 7.4). Lystes were centrifuged at 750 × g for 10 min. The supernatant was kept, and the pellet was re-suspended and centrifuged. The second supernatant was combined with the previous one, and the pellet was saved as the nuclear fraction. The pooled supernatant was centrifuged at 20,000 × g for 10 min. The pellet was saved, the supernatant was centrifuged at 105,000 × g for 1 h, and the resulting supernatant was saved as the cytosolic fraction. The 20,000 × g pellet from above was re-suspended in homogenizing buffer and centrifuged at 20,000 × g for 10 min. The supernatant was discarded, and the pellet was re-suspended and layered over 36 ml of 27% (v/v) Percoll and centrifuged at 20,000 × g for 90 min. The lysosomal band in the bottom 1–2 ml was collected and centrifuged at 100,000 × g for 1 h, and the turbid layer of lysosomes just above the pellet was collected. After protein quantification, the samples were used for Western blots.

AEP activity assay—Tissue homogenates or cell lysates (10 µg) were incubated in 200 µl reaction buffer (20 mM citric acid, 60 mM Na2HPO4, 1 mM EDTA, 0.1% CHAPS and 1...
mM DTT, pH 5.5) containing 20 μM AEP substrate Z-Ala-Ala-Asn-AMC (Bachem). AMC released by substrate cleavage was quantified by measuring at 460 nm in a fluorescence plate reader at 37 °C in kinetic mode.

**AEP cleavage assay**—HEK293 cells were transfected with GST- AEP WT/S226A/S226D or co-transfected with GST-Tau and AEP WT/S226A/S226D. 48 h after transfection, the cells were lysed in buffer (50 mM sodium citrate, 5 mM DTT, 0.1% CHAPS, 0.5% Triton X-100, 1mM EDTA, and 60 mM Na₂HPO₄, pH 5.5) and centrifuged for 15 min at 14,000g at 4 °C. The supernatants were then incubated at 37 °C for 0, 5, 10, 15, 30, and 60 min. The samples were then boiled in 1× SDS loading buffer and analyzed by immunoblotting.

**Cleavage of Tau by SRPK2-phosphorylated AEP**—HEK293 cells were co-transfected with GST-AEP and Myc-SRPK2 WT/KD. 48 h after transfection, cell lysates were prepared in buffer (50 mM sodium citrate, 5 mM DTT, 0.1% CHAPS, 0.5% Triton X-100, 1mM EDTA, and 60 mM Na₂HPO₄, pH 5.5). AEP pS226 antibody was used to test the phosphorylation. The cell lysates were incubated with recombinant Tau in buffer (50 mM sodium citrate, 5 mM DTT, 0.1% CHAPS, 1mM EDTA, and 60 mM Na₂HPO₄, pH 5.5) at 37 °C for 0, 5, 10, 15, 30, and 60 min. The samples were then boiled in 1× SDS loading buffer and analyzed by immunoblotting.

**HeLa cell synchrony**—HeLa cells were cultured in high-glucose DMEM added with 10% fetal bovine serum (FBS) and penicillin (100 units/ml) - streptomycin (100 µg/ml) (all from Hyclone). Cell were synchronized by the double thymidine block protocol (Rao and Johnson, 1970) (first block 18 hr, release 10 hr, second block 18 hr). Cells were then extracted at different stages of cell cycle as described (Gui et al., 1994; Lew et al., 1991): HeLa cells quickly entered S phase after release. They were in G2 8–10 hours after release and entered M phase 10–12 hours after release. They were predominantly in G1 until reentering S phase 18–20 hours after release.

**Immunoprecipitation and Western blot analysis**—Cells were washed with ice-cold PBS and lysed in Co-immunoprecipitation (Co-IP) buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 5 mM EGTA, 15 mM MgCl₂, 60 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1 mM NaF, 0.1 mM benzamide, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM PMSF) at 4 °C for 2 h with rotation. Immunoprecipitated proteins were separated by SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was blocked with TBS containing 5% nonfat milk and 0.1% Tween 20 (TBST) at RT for 1 h, followed by the incubation with primary antibody at 4 °C overnight, and with the secondary antibody at RT for 1 h. After washing with TBST, the membrane was developed using the enhanced chemiluminescent (ECL) detection system.

**Immunohistochemistry**—Immunohistochemistry was performed by using the peroxidase protocol. Briefly, tissue sections were deparaffinized in xylene, hydrated through descending ethanol, and endogenous peroxidase activity was eliminated by incubation in 3% hydrogen peroxide in methanol for 5 min. After antigen-retrieval in boiling sodium citrate buffer (10
mM), the sections were incubated with primary antibodies for overnight at 4 °C. The signal was developed using Histostain-SP kit (Invitrogen). For the double immunofluorescence staining, the sections were incubated overnight at 4 °C with a mixture of antibodies. After being washed with TBS, the sections were incubated with a mixture of Alexa Fluor 488- and 568-coupled secondary antibodies (Invitrogen) for detection. Images were acquired through Confocal (Olympus FV1000).

**Quantification of colocalization**—Pearson’s coefficient was used to analyze the colocalization in dual-labeled IF images. It is a standard statistical analysis to measure the strength of a linear relationship between two variables. We used ImageJ to perform quantitative analysis of confocal images. Pearson’s correlation coefficients were quantified by the ‘Colocalization’ tool in the ImageJ. The values are set between 0 and 1 (1: colocalization, 0: no co-localization).

**Aβ Plaque Staining**—Amyloid plaques were stained with Thioflavin-S. Deparaffinized and hydrated sections were incubated in 0.25% potassium permanganate solution for 20 min, rinsed in distilled water, and incubated in bleaching solution containing 2% oxalic acid and 1% potassium metabisulfite for 2 min. After rinsing in distilled water, the sections were transferred to blocking solution containing 1% sodium hydroxide and 0.9% hydrogen peroxide for 20 min. The sections were incubated for 5 s in 0.25% acidic acid, then washed in distilled water and stained for 5 min with 0.0125% Thioflavin-S in 50% ethanol. The sections were washed with 50% ethanol and placed in distilled water. Then the sections were covered with a glass cover using mounting solution. Quantitative assessment of plaque areas was done using Image J software. Briefly, the images were normalized and an automatic thresholding on the basis of the entropy of the histogram was used to identify the plaques. The pictures were converted to a binary, and then the plaque number and the plaque area were calculated.

**Electron Microscopy**—After deep anesthesia, mice were perfused transcardially with 2% glutaraldehyde and 3% paraformaldehyde in PBS. Hippocampal slices were postfixed in cold 1% OsO4 for 1 h. Samples were prepared and examined using standard procedures. Ultrathin sections (90 nm) were stained with uranyl acetate and lead acetate and viewed at 100 kV in a JEOL 200CX electron microscope. Synapses were identified by the presence of synaptic vesicles and postsynaptic densities.

**Stereotaxic injection of lentivirus in mouse CA1**—Mice were anesthetized with isoflurane, and lentivirus (LV, 3 µl with similar titers >1×10⁹ IU/ml) were delivered at a rate of 0.3 µl/min (anteroposterior (AP) −2.2 mm, mediolateral (ML) ±1.7 mm, dorsoventral (DV) −1.6mm). Mice were assigned into gender- and age-matched treatment groups using a randomized block design.

**Golgi stain**—Mouse brains were fixed in 10% formalin for 24 h, and then immersed in 3% potassium bichromate for 3 days in the dark. The solution was changed each day. Then the brains were transferred into 2% silver nitrate solution and incubated for 24 h in the dark. Vibratome sections were cut at 60 µm, air dried for 10 minutes, dehydrated through 95% and 100% ethanol, cleared in xylene and coverslipped.
**Electrophysiology**—Acute hippocampal transversal slices were prepared from different ages of WT, 5XFAD, 3xTg mice injected with lentivirus as previously described (Zhang et al., 2014; Zhang et al., 2015). Briefly, mice were anaesthetized with isoflurane, decapitated, and their brains dropped in icecold artificial cerebrospinal fluid (a-CSF) containing 124 mM NaCl, 3 mM KCl, 1.25 mM NaH$_2$PO$_4$, 6.0 mM MgCl$_2$, 26 mM NaHCO$_3$, 2.0 mM CaCl$_2$, and 10 mM glucose. Hippocampi were dissected and cut into 400-μm thick transverse slices with a vibratome. After incubation at room temperature (23–24°C) in a-CSF for 60–90 min, slices were placed in a recording chamber (RC-22C, Warner Instruments) on the stage of an up-right microscope (Olympus CX-31) and perfused at a rate of 3 ml per min with a-CSF (containing 1 mM MgCl$_2$) at 23–24°C. A 0.1 MΩ tungsten monopolar electrode was used to stimulate the Schaffer collaterals. The field excitatory post-synaptic potentials (fEPSPs) were recorded in CA1 stratum radiatum by a glass microelectrode filled with a-CSF with resistance of 3–4 MΩ. The stimulation output (Master-8; AMPI, Jerusalem) was controlled by the trigger function of an EPC9 amplifier (HEKA Elektronik, Lambrecht, Germany). fEPSPs were recorded under current-clamp mode. Data were filtered at 3 kHz and digitized at sampling rates of 20 kHz using Pulse software (HEKA Elektronik). The stimulus intensity (0.1 ms duration, 10–30 μA) was set to evoke 40% of the maximum f-EPSP and the test pulse was applied at a rate of 0.033 Hz. LTP of fEPSPs was induced by 3 theta-burst-stimulation (TBS), it is 4 pulses at 100 Hz, repeated 3 times with a 200-ms interval). The magnitudes of LTP are expressed as the mean percentage of baseline fEPSP initial slope.

**Morris Water maze**—Different ages of WT, 5XFAD, and 3xTg mice injected with the various lentiviruses were trained in a round, water-filled tub (52 inch diameter) in an environment rich with extra maze cues as described previously (Zhang et al., 2014). Each subject was given 4 trials/day for 5 consecutive days with a 15-min intertrial interval. The maximum trial length was 60 s, and if subjects did not reach the platform in the allotted time, they were manually guided to it. Following the 5 d of task acquisition, a probe trial was presented, during which time the platform was removed and the percentage of time spent in the quadrant that previously contained the escape platform during task acquisition was measured over 60 s. All trials were analyzed for latency and swim speed by means of MazeScan (Clever Sys, Inc.).

**Contextual fear conditioning**—The ability to form and retain an association between an aversive experience and environmental cues was tested with a standard fear conditioning paradigm that occurs over a period of 3 d. Mice were placed in the fear conditioning apparatus (7” W, 7” D × 12” H, Coulbourn) composed of Plexiglass with a metal shock grid floor and allowed to explore the enclosure for 3 min. Following this habituation period, 3 conditioned stimulus (CS)-unconditioned stimulus (US) pairings were presented with a 1-min intertrial interval. The CS was composed of a 20-s, 85-dB tone and US was composed of 2 s of a 0.5-mA footshock, which was co-terminate with each CS presentation. One minute following the last CS-US presentation, mice were returned to their home cage. On day 2, the mice were presented with a context test, during which subjects were placed in the same chamber used during conditioning on day 1 and the amount of freezing was recorded via a camera and the software provided by Coulbourn. No shocks were given during the context test. On day 3, a tone test was presented, during which time subjects were exposed
to the CS in a novel compartment. Initially, animals were allowed to explore the novel context for 2 min. Then the 85-db tone was presented for 6 min and the amount of freezing behavior was recorded.

QUANTIFICATION AND STATISTICAL ANALYSIS

All data are expressed as mean ± s.e.m. from three or more independent experiments, and the level of significance between two groups was assessed with Student’s t-test. For more than two groups, one-way ANOVA followed by LSD post hoc test was applied. A value of p < 0.05 was considered to be statistically significant.

DATA AND SOFTWARE AVAILABILITY

Raw data have been deposited to Mendeley Data and are available at http://dx.doi:10.17632/xwcyrwp8y8k.1

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References


Highlights

SRPK2 phosphorylates serine 226 on delta-secretase in AD barins
Delta-secretase phosphorylation escalates its enzymatic activities
Phosphorylated delta-secretase provokes AD-like pathology
Unphosphorylate delta-secretase restores cognitive functions

Wang et al. show that delta-secretase phosphorylation by SRPK2 promotes its cytoplasmic translocation and enzymatic activities. Unphosphorylate mutant rescues AD-like pathologies, implicating its potential therapeutic role in AD.
Figure 1. SRPK2 phosphorylates delta-secretase in vitro and in vivo
A. In vitro kinase assay. His-AEP was incubated with immunoprecipitated GST-SRPK2 WT or GST-SRPK2 KD in the presence of [$\gamma^{32}$P]-ATP. The reaction products were separated in SDS-PAGE and analyzed by autoradiography. Purified GST-Tau was used as positive control. The protein inputs were shown (lower panel). Phosphorylation-to-protein levels ratio was presented for AEP and Tau (right).
B. SRPK2 phosphorylates delta-secretase in intact cells. mGST-delta-secretase was co-transfected with SRPK2 wild-type or KD respectively into HEK293 cells. After 48 h, mGST-delta-secretase was pulled down and analyzed with anti-p-Serine/Threonine antibody (1st panel). Expressions of pulled-down...
GST-AEP (2nd panel) and Myc-SRPK2 (3rd panel) in cell lysates were also shown. **C.** Western blot showing phosphorylation of full-length and mature delta-secretase by SRPK2 WT and KD in HEK293 cells. Phosphorylation ratio was presented (right). **D.** S226 in delta-secretase is the major phosphorylation residue. Schematic diagrams of the potential SRPK2 phosphorylation sites in delta-secretase (top). In vitro kinase assay showing phosphorylation of WT and mutated delta-secretase (S to A, unphosphorylated mutate) by SRPK2 (upper panel). The inputs were shown (lower panel). **E.** Western blot analysis of phosphorylation level of WT and mutated delta-secretase after co-transfection with SRPK2 WT or KD. See also Figure S1.
Figure 2. Delta-secretase phosphorylation by SRPK2 in neurons and AD brains

A. Co-localization between SRPK2 pT492 and delta-secretase in rat primary cortical neurons (DIV 15) treated with 2 or 20 µM of pre-aggregated Aβ for 16 h. B. Co-localization between SRPK2 and delta-secretase pS226 in rat primary cortical neurons (DIV 15) treated with 2 or 20 µM of pre-aggregated Aβ for 16 h. A-B. Confocal pictures showed co-localization. Scale bars, 5 µm. C-D. Quantification of SRPK2 pT492 with delta-secretase (C) and SRPK2 with delta-secretase pS226 (D) co-localization. Data shown as mean ± s.e.m. (n=20–28 cells per group; *P<0.05; student’s t-test). E. Knockdown of SRPK2 reduces delta-secretase phosphorylation on S226 induced by Aβ. Western blot was
conducted from primary cultures infected with virus expressing control or SRPK2 shRNA. 

**F-G.** Western blot analysis of phosphorylated delta-secretase and SRPK2 in AD brains compared with age-matched healthy controls. Western blot data in **E-G** are representative of three independent experiments. See also Figure S2.
Figure 3. Phosphorylated delta-secretase on S226 is selectively translocated from neuronal lysosomes to the cytoplasm

A. Immunofluorescent staining. Co-localization between GST and LAMP1 in HEK293 cells transfected with GST-tagged WT and mutated delta-secretase. Confocal pictures showing co-localization between GST and LAMP1 were presented. Scale bars, 5 µm.

B. Co-localization between delta-secretase pS226 and LAMP1 in rat primary cortical neurons (DIV 15) treated with 2 or 20 µM of pre-aggregated Aβ for 16 h. Confocal pictures showed co-localization between delta-secretase pS226 and LAMP1. Scale bars, 5 µm.

C-D. Quantification of GST and LAMP1 (C) and pS226 and LAMP1 (D) co-localization was...
shown. Data shown as mean ± s.e.m. (n=15–18 cells per group; *P<0.05; student’s t-test). E. Phospho-S226 mediates delta-secretase cytosolic translocation. SH-SY5Y cells were treated with different concentrations of Aβ, and then were subjected to subcellular fractionation, followed by western blot. LAMP1 was used as specific marker for lysosomal fraction. Tubulin was applied as loading control for cytoplasm fraction. F. SRPK2-phosphorylated delta-secretase translocates into the cytoplasm from the lysosomes. SH-SY5Y cells were transfected with control siRNA or si-SRPK2, followed by Aβ (10 µM) treatment for different time points. The lysosomal and cytosolic fractions were prepared and analyzed by immunoblotting. G, Aβ triggers delta-secretase S226 phosphorylation escalation and the cytoplasm translocation. Immunofluorescent staining of SH-SY5Y cells with anti-p-S226/SRPK2/LAMP1 after Aβ treatment. Scale bars, 10 µm. H-I, Quantification of AEP pS226 intensity (H) and quantification of AEP pS226 and LAMP1 co-localization (I) was shown as Pearson’s coefficient. Data shown as mean ± s.e.m. (n=16–18 cells per group; *P<0.05; student’s t-test). See also Figure S3.
Figure 4. Delta-secretase phosphorylation on S226 by SRPK2 increases its enzymatic activity
A-C. Delta-secretase activity assay of HEK293 cell lysates. HEK293 cells were co-
transfected with delta-secretase and SRPK2 WT/KD (A), or transfected with SPRK2
WT/KD (B), or WT and mutated delta-secretase (C). Data shown as mean ± s.e.m. of three
independent experiments.

D-E. Western blot analysis of autocatalytic process of WT/mutated delta-secretase (D) and cleavage of Tau by WT/mutated delta-secretase (E) after incubation in pH5.5 reaction buffer at 37°C. Truncated delta-secretase or tau bands were
quantified using densitometry. After being normalized with total delta-secretase or tau
levels, the relative levels were plotted against reaction times. Each curve (D. lower; E. right)

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was fitted with GraphPad Prism 5 using the Boltzmann sigmoidal function. Data are representatives of four independent experiments. See also Figure S4.
Figure 5. Delta-secretase S226D in young 3xTg mice hippocampal CA1 accelerates the onset of AD pathogenesis and worsens cognitive dysfunctions

A. Delta-secretase S226D escalates delta-secretase and induces APP and Tau cleavage by delta-secretase. Hippocampal CA1 tissues from lentiviruses (control, delta-secretase WT, or S226D) injected 3xTg mice were analyzed by western blot (n = 4 mice per group). Molecular weight of no-tagged exogenous delta-secretase is similar as endogenous delta-secretase. B. Delta-secretase S226D increases its activity. Data represent mean ± s.e.m. of 3 mice per group (*P < 0.05, one-way ANOVA). C. Delta-secretase S226D stimulates the early formation of amyloid plaques in 3xTg mice CA1. Thioflavin S staining detection of senile plaques in CA1 (left, scale bar, 50 µm). Quantitative analysis of plaque number and
Plaque analysis data represent mean ± s.e.m. of 13–15 sections from 3 mice in each group (*P < 0.05, one-way ANOVA). D. Tau pathology is promoted by delta-secretase S226D. IHC staining with anti-p-Tau antibodies. The brain sections were immunostained with AT8 (upper) and AT100 (middle). Scale bar, 50 µm. AT-8 and AT100 immuno-reactivity quantification (lower, mean ± s.e.m.; 16–18 sections from 3 mice, *P < 0.05, one-way ANOVA). E. Delta-secretase S226D decreases dendritic spine density. Top, Golgi staining was conducted on brain sections from LV-Ct/ delta-secretase WT/AEP S226D treated apical dendritic layer of the CA1 region. Scale bar, 5 µm. Bottom, quantification of spine density represent mean ± s.e.m. of 9–12 sections from 3 mice in each group. (*P < 0.05, one-way ANOVA). F. Electrophysiology analysis. Delta-secretase S226D expression in CA1 worsened the LTP defects in 3xTg mice. The ratio of paired pulses in different groups (mean ± s.e.m.; n = 6 in each group; *P < 0.05 compared with 3xTg-Ct, one-way ANOVA) (left). LTP of fEPSPs (mean ± s.e.m.; n = 6 in each group; *P < 0.05 3xTg-S226D vs. 3xTg-Ct, # P < 0.05 3xTg-S226D vs. 3xTg-AEP WT, one-way ANOVA) (right). G&H. Morris Water Maze analysis of cognitive functions. Delta-secretase S226D expression in the CA1 exacerbated the learning and memory dysfunctions in 3xTg mice (mean ± s.e.m.; n = 8–10 mice per group; *P < 0.05, one-way ANOVA). See also Figure S5.
Figure 6. Delta-secretase S226A ameliorates the pathologies and behavioral defects in 5XFAD mice

A. S226A mutation reduces delta-secretase and APP and Tau fragments cleaved by delta-secretase. Hippocampal CA1 tissues from lentivirus (control, WT, or delta-secretase S226A) injected 5XFAD mice were analyzed by immunoblotting with various antibodies (n = 4 mice per group). Molecular weight of no-tagged exogenous delta-secretase is similar as endogenous delta-secretase.

B. S226A mutation reduces delta-secretase enzymatic activity. Data represent mean ± s.e.m. of 3 mice per group (*P < 0.05, **P < 0.01, one-way ANOVA).

C-D. S226A mutant decreases amyloid plaques and Aβ in 5XFAD mice CA1.

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Quantification of number and surface area of amyloid plaques (right panels). Plaque analysis data represent mean ± s.e.m. of 13–15 sections from 3 mice in each group (*P < 0.05, **P < 0.01, one-way ANOVA). Scale bar, 50 µm. D. IHC staining of Aβ in the hippocampus (left panels, scale bar, 50 µm). Quantitative analysis of Aβ immunoreactivity (right panel). Data represent mean ± s.e.m. of 12–16 sections from 3 mice in each group (*P < 0.05, one-way ANOVA). E. S226A mutant increases the spine density. Scale bar, 5 µm. Golgi staining was conducted on brain sections from CA1 regions of 5XFAD mice (mean ± s.e.m.; n = 5; *p < 0.05, one-way ANOVA). F. Electrophysiology analysis. S226A mutant increased ratio of paired pulses (left) and rescued the LTP defects in 5XFAD mice (right) (mean ± s.e.m.; n = 6 in each group; *P < 0.05 5XFAD-S226A vs. 5XFAD-Ct, # P < 0.05 5XFAD-S226A vs. 5XFAD-WT, one-way ANOVA). Shown traces are representative fEPSPs recorded before (black) and 60 minutes after (red) TBS. G-I, Morris Water Maze (G&H) and Conditional Fear Conditioning (CFC) analysis of cognitive functions. S226A mutant in CA1 rescues the learning and memory impairments in 5XFAD mice (mean ± s.e.m.; n =9–10 mice per group; *P< 0.05, one-way ANOVA). See also Figure S6.