

科技部補助專題研究計畫成果報告 期末報告

探討路易氏體失智症之AMPK-Sirt1訊息傳遞在A β 與 α -
synuclein蛋白神經毒性加成效應中可能扮演的角色(第2年)

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計畫主持人：賴德仁
共同主持人：何應瑞、林志立
計畫參與人員：碩士班研究生-兼任助理：張雁婷
碩士班研究生-兼任助理：蔡善格
碩士班研究生-兼任助理：謝佳玲
博士班研究生-兼任助理：黃玟儂
博士後研究-博士後研究：李欣樺

報告附件：出席國際學術會議心得報告

中華民國 106 年 10 月 20 日

中文摘要：路易氏體失智症是一種常見的老年認知障礙疾病，雖然目前並不清楚這個疾病的成因，但已經確認患者腦內 α -synuclein 造成的路易氏體沈積是最重要的病理特徵。令人感到興趣的是，此症患者其腦內也經常發現在阿茲海默症中才會出現的 A β 沈積，以及分析路易氏體成分也會發現 α -synuclein 與 A β 這兩個蛋白是經常共位聚集的，但其原因目前仍不明。事實上越來越多的證據已指出，若路易氏體失智症在疾病的發展過程中其與阿茲海默類似的症狀越明顯，則此症的預後便越不佳。因此，我們提出一個假說，認為 A β 會透過抑制 AMPK 的訊息傳遞，來影響 α -synuclein 的後修飾、聚集及廓清作用，並增加氧化壓力傷害來惡化路易氏體失智症的疾病進展。而我們的研究結果則發現，A β 確實可促進 α -synuclein 聚集並協同性的增強其神經毒性，此外 A β 也能透過減弱細胞自噬的活性來降低 α -synuclein 被分解的速率，並經由抑制 AMPK 及 Sirt1 訊息傳遞來加速神經細胞老化。我們認為這些研究結果將能解釋 A β 在 DLB 病程進展中所扮演的角色，並評估藉由增進細胞自噬降解的方式是否能清除已聚集的 α -synuclein，我們希望這些研究結果能有助於在未來發展出新型的 DLB 治療策略。

中文關鍵詞：阿茲海默症、A β 蛋白、細胞自噬、路易氏體失智症、 α -synuclein

英文摘要：Dementia with Lewy bodies (DLB) is a common cause of geriatric cognitive impairments. The underlying biology of DLB is complex, but the presence of α -synuclein aggregation containing Lewy body is most important features. It is interesting that many DLB cases are often displayed amyloid β (A β) depositions that are associated with Alzheimer's disease (AD). Additionally, α -synuclein and A β are also frequently co-localized in Lewy body. The reason for this overlap is unknown. Actually, the greater the AD pathology in DLB patients, the more severe with disease progression in DLB. As a result, we postulate A β may contribute to α -synuclein-induced neurotoxicity by protein modification, aggregation, clearance and oxidative stress in DLB. According to our results, A β can promote α -synuclein aggregation, and enhances neurotoxicity in a synergistic effect. Moreover, A β also decreased the degradation of aggregated α -synuclein by interfering autophagy process, and induced neuronal cell senescence by downregulating of AMP-activated protein kinase (AMPK) and Sirt1 anti-aging signaling. We expect our results can confirm and extend the contributing role of A β in DLB pathogenesis, and demonstrates the confluence of aggregated α -synuclein clearance by rescue of autophagic degradation pathways. Based on these findings, we hope to provide novel insights in DLB therapeutic strategies in future.

英文關鍵詞：Alzheimer's disease (AD); amyloid β (A β); autophagy; Dementia with Lewy bodies (DLB); α -synuclein.

科技部補助專題研究計畫成果報告

(期中進度報告/期末報告)

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蛋白神經毒性加成效應中可能扮演的角色

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計畫主持人：賴德仁 教授

共同主持人：何應瑞 教授

共同主持人：林志立 副教授

計畫參與人員：李欣樺博士後研究員

黃玟儂博士班研究生

張雁婷、蔡善格、謝佳玲碩士班研究生

本計畫除繳交成果報告外，另含下列出國報告，共 1 份：

執行國際合作與移地研究心得報告

出席國際學術會議心得報告

出國參訪及考察心得報告

中 華 民 國 106 年 10 月 15 日

中、英文摘要及關鍵詞(keywords)

摘要(中文)

路易氏體失智症是一種常見的老年認知障礙疾病，雖然目前並不清楚這個疾病的成因，但已經確認患者腦內 α -synuclein 造成的路易氏體沈積是最重要的病理特徵。令人感到興趣的是，此症患者其腦內也經常發現在阿茲海默症中才會出現的 $A\beta$ 沈積，以及分析路易氏體成分也會發現 α -synuclein 與 $A\beta$ 這兩個蛋白是經常共位聚集的，但其原因目前仍不明。事實上越來越多的證據已指出，若路易氏體失智症在疾病的發展過程中其與阿茲海默類似的症狀越明顯，則此症的預後便越不佳。因此，我們提出一個假說，認為 $A\beta$ 會透過抑制 AMPK 的訊息傳遞，來影響 α -synuclein 的後修飾、聚集及廓清作用，並增加氧化壓力傷害來惡化路易氏體失智症的疾病進展。而我們的研究結果則發現， $A\beta$ 確實可促進 α -synuclein 聚集並協同性的增強其神經毒性，此外 $A\beta$ 也能透過減弱細胞自噬的活性來降低 α -synuclein 被分解的速率，並經由抑制 AMPK 及 Sirt1 訊息傳遞來加速神經細胞老化。我們認為這些研究結果將能解釋 $A\beta$ 在 DLB 病程進展中所扮演的角色，並評估藉由增進細胞自噬降解的方式是否能清除已聚集的 α -synuclein，我們希望這些研究結果能有助於在未來發展出新型的 DLB 治療策略。

關鍵字：阿茲海默症、 $A\beta$ 蛋白、細胞自噬、路易氏體失智症、 α -synuclein

Abstract (English)

Dementia with Lewy bodies (DLB) is a common cause of geriatric cognitive impairments. The underlying biology of DLB is complex, but the presence of α -synuclein aggregation containing Lewy body is most important features. It is interesting that many DLB cases are often displayed amyloid β ($A\beta$) depositions that are associated with Alzheimer's disease (AD). Additionally, α -synuclein and $A\beta$ are also frequently co-localized in Lewy body. The reason for this overlap is unknown. Actually, the greater the AD pathology in DLB patients, the more severe with disease progression in DLB. As a result, we postulate $A\beta$ may contribute to α -synuclein-induced neurotoxicity by protein modification, aggregation, clearance and oxidative stress in DLB. According to our results, $A\beta$ can promote α -synuclein aggregation, and enhances neurotoxicity in a synergistic effect. Moreover, $A\beta$ also decreased the degradation of aggregated α -synuclein by interfering autophagy process, and induced neuronal cell senescence by downregulating of AMP-activated protein kinase (AMPK) and Sirt1 anti-aging signaling. We expect our results can confirm and extend the contributing role of $A\beta$ in DLB pathogenesis, and demonstrates the confluence of aggregated α -synuclein clearance by rescue of autophagic degradation pathways. Based on these findings, we hope to provide novel insights in DLB therapeutic strategies in future.

Key words: Alzheimer's disease (AD); amyloid β ($A\beta$); autophagy; Dementia with Lewy bodies (DLB); α -synuclein.

前言

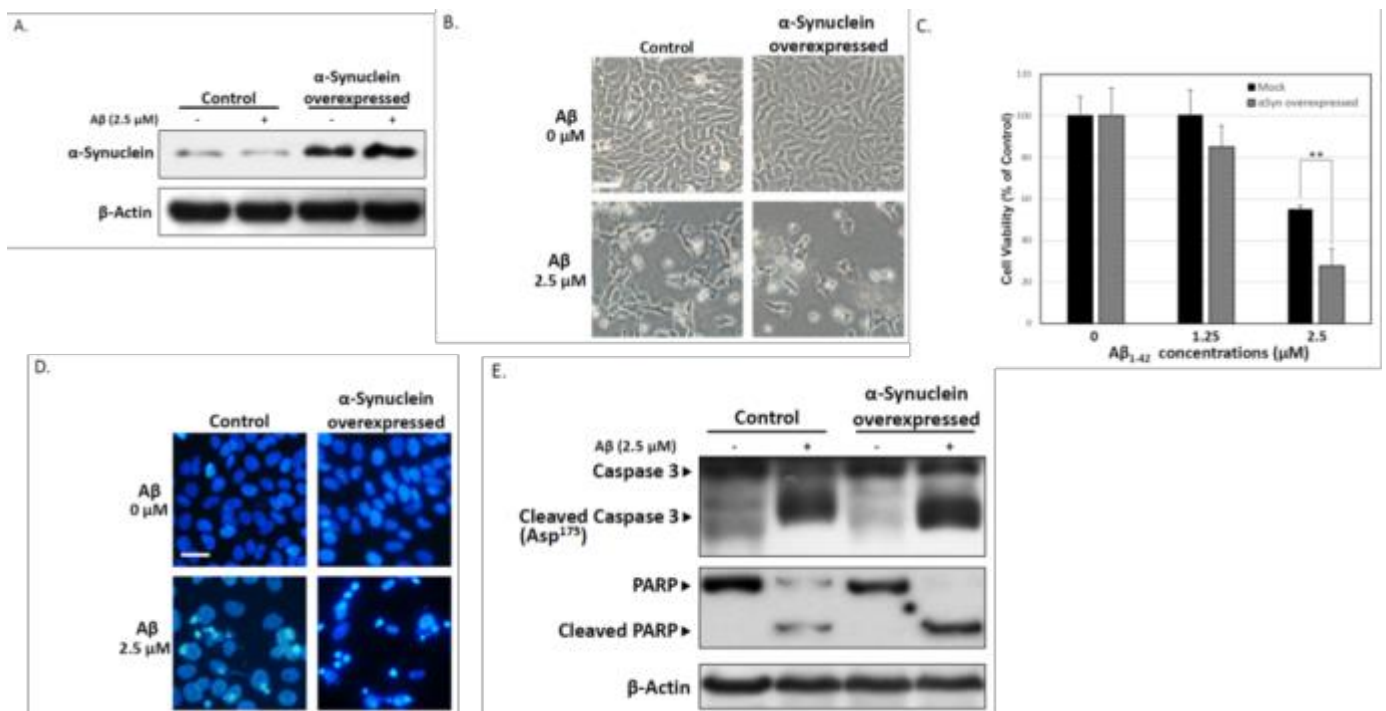
路易氏體失智症(Dementia with Lewy bodies, DLB) 是一種中樞神經退化性疾病，除認知功能缺損外，也常伴隨動作障礙的症狀，因此是一個臨床表現重疊於巴金森氏症(Parkinson's disease, PD)與阿茲海默症(Alzheimer's disease, AD)之間的疾病。Lewy bodies 因此被認為是 DLB 最主要的病理特徵，在 DLB 病程的早期通常會先同時或分別在皮質(cortical)與皮質下(subcortical)之腦區產生，而靠近這些 Lewy bodies 的腦組織便會受其影響而逐漸衰弱凋亡，並因此引發出相對應的病徵。已知 Lewy body 的構成物中包含著數種蛋白質，包括 ubiquitin、amyloid β ($A\beta$)與 neurofilament 等，但最主要成分是 α -synuclein。 α -synuclein 是由 SNCA 基因所轉譯出的一個 140 個胺基酸的蛋白質，在某些情況下會發生聚集沈積。在中樞神經系統(central nervous system, CNS)中， α -synuclein 主要表現在大腦 substantia nigra、striatum 及 hippocampus 等區域，其明確生理功能至今仍不明，推測可能與神經突觸傳導功能的調節有關。奇怪的是正常的 α -synuclein 並不會表現出任何聚集沈積或誘發神經毒性的傾向，需要經由某些目前尚未知曉的因子影響後才會被誘發聚集並形成 Lewy body。事實上 α -synuclein 最早反而被認為是 AD 相關的致病因子，直到 1997 年在歐洲幾個 familial PD 中發現 α -synuclein 的突變會造成自發性的聚集並形成 Lewy body，自此 α -synuclein 便被歸類於 PD 的主要致病因子。截至目前為止 α -synuclein 上已發現 3 個突變位，包括 A53T、A30P 與 E46K 等，但這些突變位都僅於少數的家族中被發現，是故因 α -synuclein 突變而致使 Lewy body 產生的病例其實僅只佔了極為少數，且這個機制也只能解釋遺傳性的 PD 或 DLB。因此對於絕大多數 sporadic DLB 而言，其 α -synuclein 究竟是受何種因子影響而傾向於發生聚集形成 Lewy body？我們認為此問題可能要從 α -synuclein 突變以外的因子來探討，其中最有可能的機制是所謂的轉譯後修飾作用(post-translational modification)，但究竟如何影響這些修飾誘發 α -synuclein 轉變成聚集反應？這個問題對於解析 DLB 的真正病因非常關鍵。雖然導致 DLB 發病機制目前尚不清楚，但 DLB 與 AD 的關係一直都很密切。研究證實患者早期腦部之 acetylcholine 分泌量都會明顯減少，且 choline acetyltransferase 的活性也有明顯減低的現象。事實上如果分析 Lewy body 的成分，會發現其中主要構成物除了 α -synuclein 外，也會發現幾乎等量的 $A\beta$ 存在於其中，因此有學者認為 DLB 與 AD 確實存在著某種特定的關聯性。舉例來說，Colom-Cadena 等人分析 DLB 病人腦部受影響的神經元，發現 α -synuclein 的表現量與 $A\beta$ 的含量呈現高度正相關，而 PET 的影像結果亦指出 DLB 與 AD 兩者大腦其 $A\beta$ 的沈積位置其實十分相似；此外 Crew 與 Haggerty 等人在動物實驗中也發現，讓小鼠的 $A\beta$ 與 α -synuclein 同時過量表現時，會促進明顯的 Lewy body 產生並導致神經元退化，而相對僅過量表現 α -synuclein 的小鼠則沒有以上的現象；另外 $A\beta$ 也被認為可透過影響 α -synuclein 在細胞內的轉譯後修飾作用，進而改變其蛋白質構型並造成折疊錯誤，其機制可能是透過 $A\beta$ 影響某些調節酵素，導致 α -synuclein 遭受不正常的後修飾作用，包括 phosphorylation、nitration、ubiquitination 及 proteolysis 等。這些證據都指出 $A\beta$ 確實有可能參與 DLB 之 α -synuclein 的聚集過程。然而目前對於 $A\beta$ 在 DLB 疾病進展中其對 α -synuclein 的影響所知仍有限，需要更詳細的研究才能證明上的觀點。

研究目的

DLB 在所有 dementia 中其發生率僅次於 AD，但目前對 DLB 的發病原因所知甚少，迄今也沒有任何藥物可有效的治療或延緩此症，因此有必要對 DLB 的分子致病機轉進行更詳細的研究。Lewy body 是由不正常折疊的 α -synuclein 所構成，但在絕大多數的 DLB 中並未發現 α -synuclein 基因存在有遺傳缺陷，表示蛋白質突變並不是 α -synuclein 產生聚集與沈積的主要原因。而流行病學研究結果發現 DLB 與 AD 其 pathogenesis 確實具有某些密切的關聯性，再加上病理學檢查也發現 Lewy body 經常是由 α -synuclein 與 $A\beta$ 相互糾結構成，因此我們認為 $A\beta$ 可能在 α -synuclein 所誘發的神經毒性中扮演了一定程度的角色。而根據我們實驗室的初步研究，發現若僅讓神經細胞過量表達 α -synuclein，其實並不會產生明顯的不良反應，這表示 α -synuclein 本身確實無法單獨誘發神經退化的現象。此外在我們先前的研究也曾發現， $A\beta$ 會干擾 ubiquitin-proteasome 的作用，並透過抑制 AMPK-mTOR 及 AMPK-Sirt1 signaling 來降低細胞內 autophagy 與 antioxidative genes 的作用，而 $A\beta$ 這個特性很有可能會誘發 α -synuclein 的後修飾並因此影響到其細胞內聚集堆積的傾向，最終誘使 Lewy body 產生以造成神經毒性。因此本研究的主要目的，是希望深入探討 AMPK 在 $A\beta$ 與 α -synuclein 的細胞內沈積及細胞毒性中所扮演的角色，並嘗試是否能夠藉由活化或回復 AMPK-Sirt1 signaling，來抑制 α -synuclein 的聚集沈積，以減輕其所造成的神經毒性。我們希望此研究結果除了能對 α -synuclein 的生理及病理角色能更了解外，也能應用在未來發展 DLB 的預防、診斷與治療策略。

實驗結果

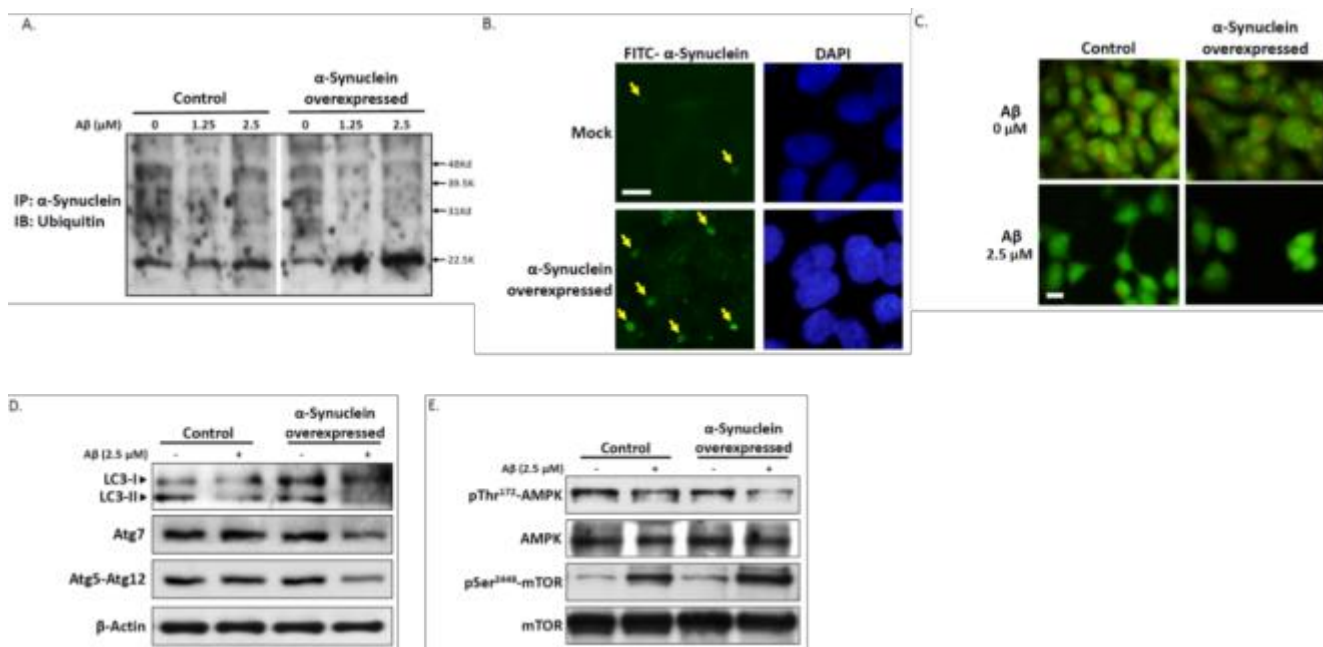
1. Overexpressed α -Synuclein enhances $A\beta$ -induced cytotoxicity in SK-N-MC human neuronal cells



(A) α -Synuclein-overexpressed cells were selected and maintained in complete medium post the pCMV-Sport6 transfection. Overexpressed cells display a significantly higher and stable levels of intracellular α -Synuclein, and is not altered by exposure to 2.5 μ M of $A\beta_{1-42}$ for 24 h. (B) No obvious morphological change was observed in α -Synuclein overexpressed cells. However, treatment with $A\beta$ markedly induced cell granules and shrinkage. (C) Effects of α -Synuclein induced-cytotoxicity by exposure to 2.5 μ M of A

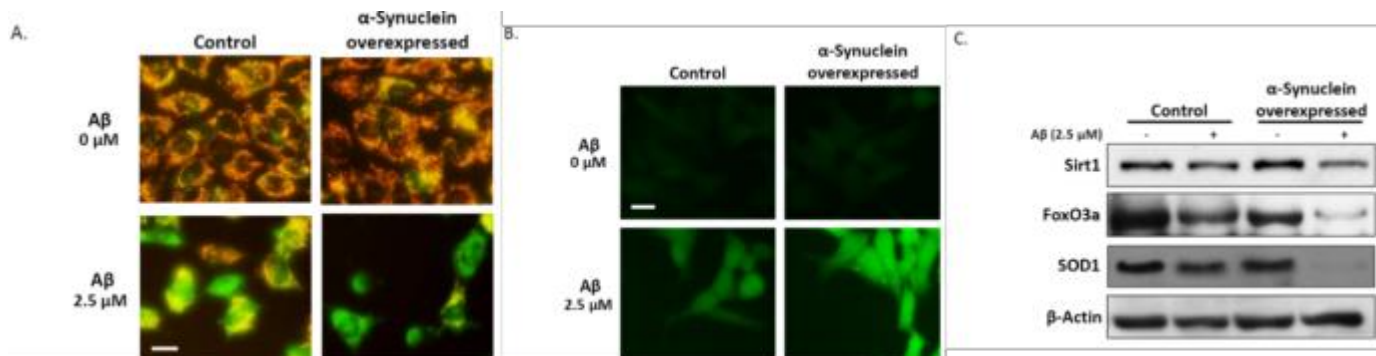
β 1-42 for 24 h, determined by the MTT assay. Treatment of A β in overexpressed α -Synuclein groups resulted in more cell death than A β only groups. Fragmented nuclei stained by DAPI (D) and caspase-3 and PARP activation (E) by immunoblotting were evaluated in A β -treated α -Synuclein overexpressed cells. Results are expressed as mean \pm SEM of three independent experiments performed in triplicates; **P < 0.01 when compared with non-treated cells by using one-way ANOVA test. Scale bar represents 50 μ m.

2. A β stimulates α -Synuclein aggregation and inhibits autophagy in α -Synuclein-overexpressed cells



(A) Immunoprecipitation results show that A β results in an increase of mono- but poly-ubiquitination of α -Synuclein. (B) Immunofluorescence analysis was performed using α -Synuclein antibody and DAPI staining. The arrows indicate the α -Synuclein-positive aggregates. (C) Acridine orange staining. Exposure to 2.5 μ M of A β for 24 h significantly decreased the formation of acridine orange-accumulating autophagic vacuoles in α -Synuclein overexpressed cells. (D) Western blots showing autophagy inhibition by monitoring LC3, Atg7, and Atg12-Atg5 conjugate in in both vector and α -Synuclein overexpressed cells. (E) Adding A β markedly suppressed phosphorylation of AMPK, and stimulated the induction of mTOR phosphorylation compared with the non-treated group. Scale bar represents 20 μ m.

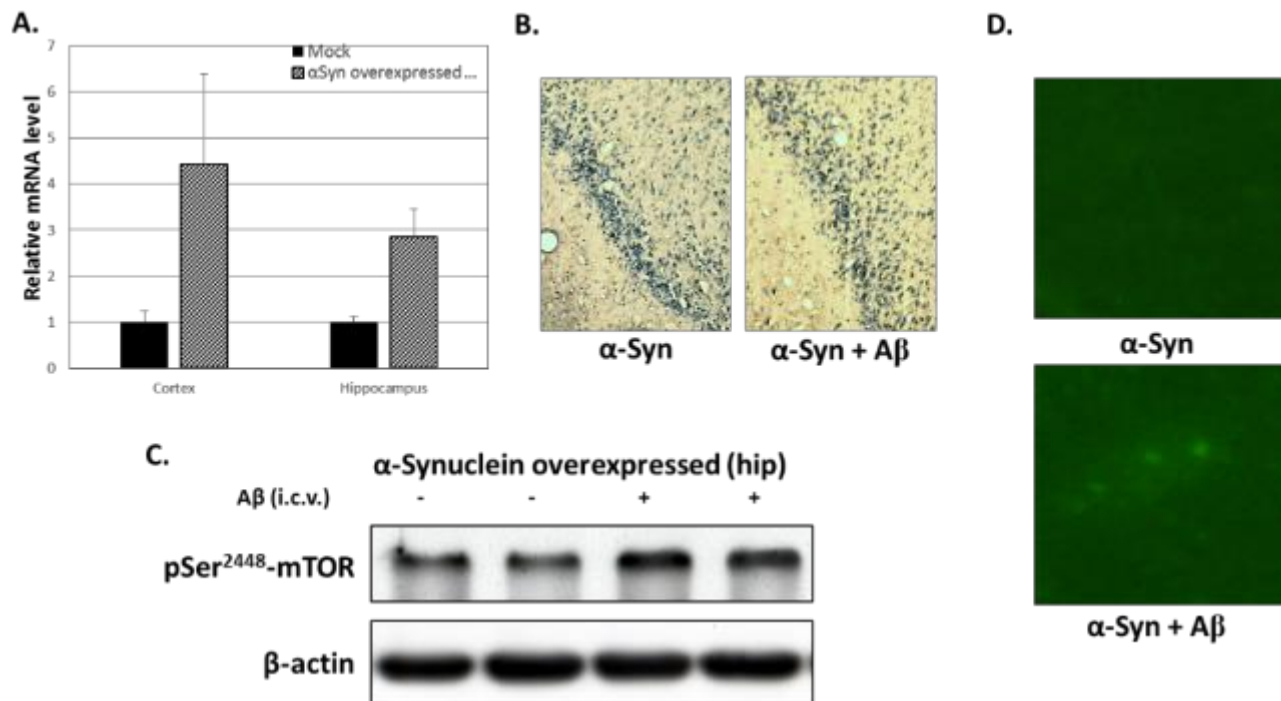
3. A β mediates mitochondrial dysfunction and antioxidative stress signaling induced by overexpression of α -Synuclein



(A) JC-1 immunofluorescent staining. Red fluorescence indicates normal mitochondrial membrane potential, and green fluorescence represents A β -induced mitochondrial dysfunction in α -Synuclein overexpressed cells. (B) Effects of α -Synuclein overexpression in exacerbating A β -induced intracellular ROS accumulation, demonstrated by dihydroethidium staining. (C) Adding A β markedly

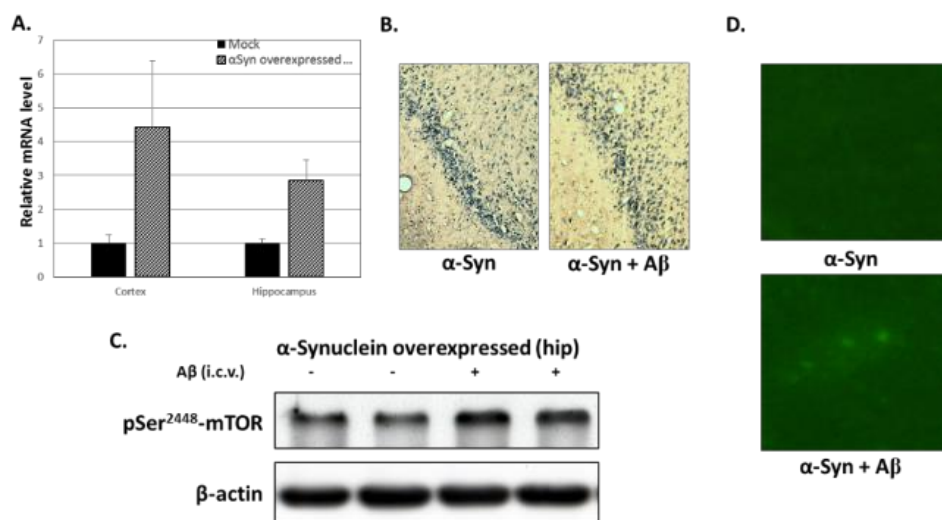
induces the reduction of Sirt1, FoxO3a and SOD1 compared with α -Synuclein non-overexpressed group. Scale bar represents 20 μ m.

4. $A\beta$ -induced hippocampal neuronal loss and mTOR activation in SD rat by i. c. v. transfection with α -Synuclein overexpression vector



(A) Intracerebroventricular (i.c.v) co-injection of A β and α -synuclein in SD rat. The hippocampus and prefrontal cortex mRNA levels of α -synuclein was measured by using real-time RT-PCR, and the results are presented as means \pm SEM. (B) Histopathological analysis revealed a loss of hippocampal pyramidal neurons of co-injection of A β and α -synuclein SD rat. (C) A β markedly stimulated the mTOR phosphorylation, revealing A β may inhibit autophagic process in rat brain. (D) Immunofluorescence results showed A β -induced α -synuclein aggregation in SD rat brain.

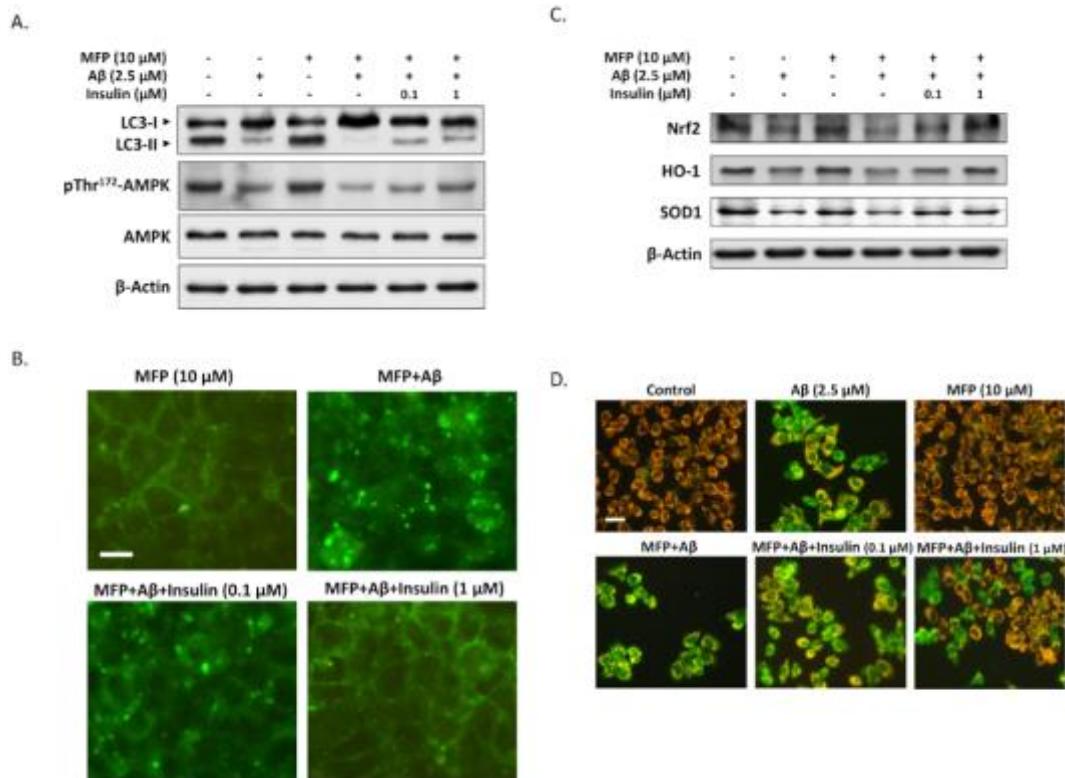
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5. Treatment of insulin suppresses A β -induced α Syn aggregation and oxidative stress.



(A) Western blotting results revealed that treatment with insulin causes limited effects on increased AMPK Thr172 phosphorylation and LC3-II levels, suggesting that impaired autophagy activity cannot be fully restored by insulin. (B) Immunofluorescence analysis showed that α Syn aggregates were markedly decreased by treating with insulin in A β -treated α Syn-overexpressed cells. Scale bar represents 10 μ m. (C) Some antioxidant signaling-related proteins including Nrf2, HO-1 and SOD1 were analyzed by western blotting, and inhibitory effects of this antioxidant pathway were effectively restored by insulin treatment. (D) JC-1 immunofluorescent staining. Green fluorescence represents A β -induced mitochondrial dysfunction in α Syn-overexpressed cells by dissipation of mitochondrial membrane potential. Red fluorescence indicates that co-treatment with insulin preserves an intact mitochondrial membrane potential. Scale bar represents 20 μ m.

6. The expression of Sirt1 and SOD1 mRNA in peripheral white blood cells of DLB patients

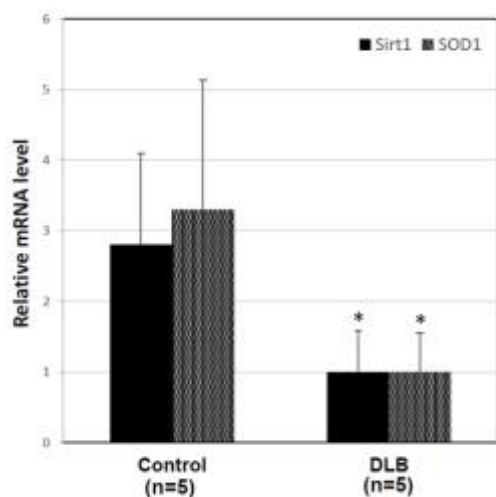


Table 1. Background characteristics of normal control and patients with DLB

Characteristics	DLB (n=5)	Control (n=5)
Age, years	75.0 ± 2.2	75.8 ± 4.8
Sex, female : male	3:2	3:2
MMSE score	19.8 ± 3.1	N/A
CASI score	66.8 ± 12.1	N/A

Values shown are means ± SD. N/A, not available.

The mRNA levels of Sirt1 and SOD1 were measured by using real-time RT-PCR, and the results are presented as means ± SEM. A detailed overview of the patient's backgrounds is summarized in Table 1. Statistical analysis was performed by using two-tailed Student's t-test. *P < 0.05 when compared with the normal control groups.

重要性及影響性

DLB 在所有退化性失智症中的發生率僅次於 AD，但目前仍缺乏有效的藥物來減緩神經元持續的退化，即使給予病人良好的照護與處置，DLB 病患的症狀終究不可避免的會持續惡化下去，因此發展機制性的策略將是治療 DLB 的關鍵。然而 DLB 的臨床特徵十分複雜，經常合併表現類似 AD 的失智症表現或類似 PD 的運動障礙，且在 biomarkers 方面的檢查包括基因、血液或腦脊髓液等方面也都沒有好的診斷指標。透過執行本計畫，我們已初步釐清 DLB 的分子致病機轉，特別是證實了 A β 在 DLB 的病程進展中所扮演的角色，並指出其所參與的詳細分子途徑及可能的預防機制，本研究結果將有助於未來開發包括 DLB 之 synucleinopathy 相關疾病的預防、診斷與治療策略，具體獲致的結果如下：

- (1) 釐清 A β 是確實會影響到 α -synuclein 的後修飾、聚集及廓清作用。
- (2) 發現 AMPK-Sirt1 signaling 確實參與在 α -synuclein 神經毒性過程中，而 A β 便是透過抑制本路徑，來促使 α -synuclein 產生變性聚集的現象。
- (3) 成功的建立了 DLB 的動物實驗模式，模擬 A β 誘發 α -synuclein 神經毒性之 DLB 致病過程。
- (4) 測量了 DLB 患者血液中 α -synuclein、autophagy 與 AMPK-Sirt1 signaling 等相關蛋白表現，並發現 AMPK-Sirt1 signaling 在 DLB 患者的 PBMC 中表現量顯著比對到組要低了許多，但詳細的臨床意義和應用則仍須更多的實驗來加以驗證。
- (5) 證實經由誘發 autophagy(例如提高神經胰島素訊息傳遞的活性)確實可作為清除已形成之 aggregated α -synuclein 之 DLB 治療策略。

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附錄：以下為計畫執行期間與本計劃內容相關發表之主要著作：

期刊論文

1. Lin CL, Cheng YS, Li HH, Chiu PY, Chang YT, Ho YJ, Lai TJ*. Amyloid- β suppresses AMP-activated protein kinase (AMPK) signaling and contributes to α -synuclein-induced cytotoxicity. *Exp. Neurol.* **275**: 84-98, 2016. (SCI) (IF=4.706, R/C=52/258; 20.2% of NEUROSCIENCES)
2. Li HH, Lin SL, Huang CN, Lu FJ, Chiu PY, Huang WN, Lai TJ*, Lin CL. miR-302 attenuates A β -induced neurotoxicity through activation of Akt signaling. *J. Alzheimers Dis.* **50**: 1083-1098, 2016. (SCI) (IF=3.731, R/C=77/258; 29.8% of NEUROSCIENCES)
3. Chiu PY, Wang CW, Tsai CT, Li HH, Lin CL, Lai TJ*. Depression in dementia with Lewy bodies: A comparison with Alzheimer's disease. *PLoS One.* **12**:e0179399, 2017. (SCI) (IF=2.806, R/C=15/64; 23.4% of MULTIDISCIPLINARY SCIENCES)
4. Chang CC, Li HH, Chang YT, Ho YJ, Hsieh JL, Chiu PY, Cheng YS, Lin CL, Lai TJ*. A β exacerbates α -synuclein-induced neurotoxicity through impaired insulin signaling in α -synuclein-overexpressed human SK-N-MC neuronal cells. *CNS Neurosci. Ther.* (In press) (SCI) (IF=3.919, R/C=47/256; 18.4% of PHARMACOLOGY & PHARMACY)

研討會論文

1. Chang YT, Huang WN, Tsai SG, Kim HG, Li HH, Lin YM, Chang CC, Lin CL, Lai TJ*. A β promotes α Syn neurotoxicity and cognitive impairment in an α Syn-overexpressed rat animal model. *11th Annual Conference of Taiwanese Society of Geriatric Psychiatry. G12, Kaohsiung, Taiwan, March 2016.*
2. Chang YT, Tsai SG, Huang WN, Kim HG, Li HH, Lin YM, Lai TJ, Lin CL. A β enhances α -Synuclein-induced neuropathology by stereotaxic ICV injections in Wistar rats. *31th Joint Annual Conference of Biomedical Science. P247, Taipei, Taiwan, March 2016.*
3. Lai TJ*, Chang YT, Li HH, Chang CC, Ho YJ, Chiu PY, Lin CL. Synergistic effects of A β on α -synuclein-induced neurotoxicity and behavioral deficits in dementia with Lewy bodies (DLB). *2016 International Psychogeriatric Association International Congress. P36, San Francisco, USA, September 2016.*
4. Lin CL, Li HH, Chang CC, Ho YJ, Chiu PY, Hsieh CL, Lai TJ*. Investigating molecular mechanisms of A β and α -synuclein synergistic effects of neurotoxicity and behavioral deficits in dementia with Lewy Bodies. *2016 International Psychogeriatric Association Asian Regional Meeting. Invited speaker, Taipei, Taiwan, December, 2016.*
5. Lai TJ*, Hsieh JL, Li HH, Huang WN, Chang CC, Kuo CY, Lin CL. Glucagon-like peptide 1 attenuates dementia with Lewy bodies (DLB) associated neurotoxicity through regulation of insulin resistance. *12th Annual Conference of Taiwanese Society of Geriatric Psychiatry. G15, Taichung, Taiwan, March 2017. (1st class reward)*
6. Lin CL, Huang CN, Li HH, Liu GY, Hung HC, Lai TJ*. Roles of ornithine Decarboxylase (ODC) in regulation of amyloid β -induced microglial neuroinflammation. *Alzheimer's Association International Conference 2017. P3-05, London, England, July 2017.*

7. Lin CL, Li HH, Kim HG, Chang CC, Chiu PY, Ho YJ, Lai TJ*. Co-expression of Oct4, Sox2, Klf4 and Nanog (KOSN) transcription factors protects against amyloid β -induced neurotoxicity by improving insulin signaling sensitivity. *Belyaev Conference 2017, Invited speaker, Novosibirsk, Russia, August, 2017.*

科技部補助專題研究計畫成果自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現（簡要敘述成果是否具有政策應用參考價值及具影響公共利益之重大發現）或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

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未達成目標（請說明，以 100 字為限）

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2. 研究成果在學術期刊發表或申請專利等情形(請於其他欄註明專利及技轉之證號、合約、申請及洽談等詳細資訊)

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DLB 在所有退化性失智症中的發生率僅次於 AD，但目前仍缺乏有效的藥物來減緩神經元持續的退化，即使給予病人良好的照護與處置，DLB 病患的症狀終究不可避免的會持續惡化下去，因此發展機制性的策略將是治療 DLB 的關鍵。然而 DLB 的臨床特徵十分複雜，經常合併表現類似 AD 的失智症表現或類似 PD 的運動障礙，且在 biomarkers 方面的檢查包括基因、血液或腦脊髓液等方面也都沒有好的診斷指標。透過執行本計畫，我們已初步釐清 DLB 的分子致病機轉，特別是證實了 A β 在 DLB 的病程進展中所扮演的角色，並指出其所參與的詳細分子途徑及可能的預防機制，本研究結果將有助於未來開發包括 DLB 之 synucleinopathy 相關疾病的預防、診斷與治療策略。

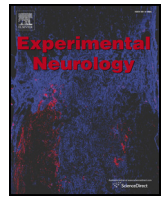
4. 主要發現

本研究具有政策應用參考價值：否 是，建議提供機關_____

(勾選「是」者，請列舉建議可提供施政參考之業務主管機關)

本研究具影響公共利益之重大發現：否 是

說明：(以 150 字為限)



Amyloid- β suppresses AMP-activated protein kinase (AMPK) signaling and contributes to α -synuclein-induced cytotoxicity



Chih-Li Lin ^{a,b}, Yu-Shih Cheng ^a, Hsin-Hua Li ^a, Pai-Yi Chiu ^{a,c}, Yen-Ting Chang ^a, Ying-Jui Ho ^{d,e}, Te-Jen Lai ^{a,e,*}

^a Institute of Medicine, Chung Shan Medical University, Taichung City, Taiwan

^b Department of Medical Research, Chung Shan Medical University Hospital, Taichung City, Taiwan

^c Department of Neurology, Show Chwan Memorial Hospital, Changhua, Taiwan

^d School of Psychology, Chung Shan Medical University, Taichung City, Taiwan

^e Department of Psychiatry, Chung Shan Medical University Hospital, Taichung City, Taiwan

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ABSTRACT

Dementia with Lewy bodies (DLB) is a neurodegenerative disorder caused by abnormal accumulation of Lewy bodies, which are intracellular deposits composed primarily of aggregated α -synuclein (α Syn). Although α Syn has been strongly implicated to induce neurotoxicity, overexpression of wild-type α Syn is shown to be insufficient to trigger formation of protein aggregates by itself. Therefore, investigating the possible mechanism underlying α Syn aggregation is essential to understand the pathogenesis of DLB. Previous studies have demonstrated that amyloid β ($A\beta$), the primary cause of Alzheimer's disease (AD), may promote the formation of α Syn inclusion bodies. However, it remains unclear how $A\beta$ contributes to the deposition and neurotoxicity of α Syn. In the present study, we investigated the cytotoxic effects of $A\beta$ in α Syn-overexpressed neuronal cells. Our results showed that $A\beta$ inhibits autophagy and enhances α Syn aggregation in α Syn-overexpressed cells. Moreover, $A\beta$ also reduced sirtuin 1 (Sirt1) and its downstream signaling, resulting in increased intracellular ROS accumulation and mitochondrial dysfunction. Our in vitro and in vivo studies support that $A\beta$ -inhibition of AMP-activated protein kinase (AMPK) signaling is involved in the neurotoxic effects of α Syn. Taken together, our findings suggest that $A\beta$ plays a synergistic role in α Syn aggregation and cytotoxicity, which may provide a novel understanding for exploring the underlying molecular mechanism of DLB.

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1. Introduction

Dementia with Lewy bodies (DLB) is the second most common progressive dementia after Alzheimer's disease (AD) (Mayo and Bordelon, 2014). The main pathological features of DLB are abnormal protein deposits inside brain neurons. These deposits, known as Lewy bodies, cause cytotoxic effects to the neuronal cells. Structurally, Lewy bodies are composed primarily of α -synuclein (α Syn), a 140-amino soluble protein encoded by the *SNCA* gene abundantly expressed in the central nervous system (Kim et al., 2014). α Syn is likely to play a role in membrane stability and neuronal plasticity; however, its exact function remains largely unknown (Schulz-Schaeffer, 2010). Although some studies have implicated that α Syn is associated with cytotoxicity in DLB pathology, overexpression of wild-type α Syn is hard to generate α Syn protein aggregates (Ko et al., 2008). Therefore, investigating the mechanism underlying α Syn aggregation is of great importance in understanding DLB. To date, research has shown three missense mutations

(A30P, E46K, A53T) present in the *SNCA* gene in causing familial synucleinopathies (Ozansoy and Basak, 2013). However, mutations in *SNCA* gene are responsible for only a very small fraction of familial cases, indicating mutated α Syn cannot be the primary cause of most DLB patients (Meeus et al., 2012). In fact, high levels of α Syn alone do not readily aggregate or form fibrils even with prolonged incubation (Hong et al., 2011). This indicates that some other pathological attributes may contribute to the process of α Syn aggregation. In addition, it is interesting that oxidative stress appears to accelerate abnormal aggregation of α Syn, supporting a cause–effect relationship between oxidative stress and α Syn aggregation in the pathogenesis of DLB (Pukass and Richter-Landsberg, 2014). However, the molecular mechanisms underlying wild-type α Syn aggregation are still largely unknown.

Although α Syn is the main component of Lewy bodies in DLB or Parkinson's disease (PD), it is originally found in senile plaques from AD patients' brains (Ueda et al., 1993). As we know, DLB shares symptoms and sometimes overlaps with AD. Actually, patients who develop DLB often have behavioral and memory symptoms of dementia like those in AD. Furthermore, most DLB patients also have concomitant AD pathology, making it difficult to distinguish between AD and DLB (Fujishiro et al., 2013). In fact, up to one-fourth of clinically-diagnosed DLB patients also develop cortical deposition of amyloid- β ($A\beta$), the

* Corresponding author at: Institute of Medicine, Chung Shan Medical University, No. 110, Sec. 1, Jianguo N. Rd., Taichung City 402, Taiwan.
E-mail address: ltj3123@ms2.hinet.net (T.-J. Lai).

most characteristic hallmark of AD (Hishikawa et al., 2003). In addition, imaging studies also show a significant increase of α Syn aggregates in human brains coexisting with A β deposits (Maetzler et al., 2009). Similarly, animal studies demonstrate A β brain deposits are highly associated with local aggregation of α Syn in Tg2576 transgenic mice (Emmer et al., 2012). These findings indicate that the presence of A β in neurons may contribute to α Syn aggregation and neurotoxicity (Pletnikova et al., 2005), but the exact nature of A β in this process is still unclear.

In addition, α Syn neurotoxicity is also linked to oxidative stress (Scarlatia and Golebiewska, 2014). Likewise, A β -induced oxidative stress has also been shown to interfere in protein folding and degradation in neuronal cells, and these pathologic features are very similar to those found in DLB (Kotzbauer et al., 2001). In fact, emerging evidence has implicated A β can suppress neuronal autophagy (Silva et al., 2011), suggesting that a reduced autophagic clearance may deteriorate the accumulation of some aggregation-prone proteins such as α Syn (Nilsson and Saido, 2014). Particularly, A β has been reported to impair the activity of AMP-activated protein kinase (AMPK) (Park et al., 2012), a kinase which plays a key role in the activation of autophagy by inhibiting activity of mammalian target of rapamycin (mTOR) (Perez-Revuelta et al., 2014). Moreover, AMPK also stimulates sirtuin 1 (Sirt1) signaling to attenuate oxidative stress (Salminen and Kaarniranta, 2012). For example, FoxO3a has been characterized as the downstream substrate of Sirt1 which forms a complex that induces ROS detoxifying enzymes such as superoxide dismutase (SOD) in response to oxidative stress (Hori et al., 2013). This Sirt1–FoxO3a axis is an evolutionarily conserved pathway that protects against oxidative stress (Salminen et al., 2013). Since α Syn neurotoxicity is reported to be associated with reduced AMPK activation (Dulovic et al., 2014), these findings raise a possibility that A β may contribute to α Syn-induced toxicity by suppression of AMPK. Particularly, increased levels of reactive oxygen species (ROS) has been suggested to induce progressive oxidative damage and aging, which ultimately leads to mitochondrial dysfunction and trigger several apoptotic pathways in the brain (Navarro et al., 2009). However, the molecular basis that links A β and α Syn-induced cytotoxicity remains unclear. Since α Syn aggregation plays a key role in DLB pathogenesis, it is crucial to understand how A β induces aggregation and cytotoxicity in α Syn.

2. Materials and methods

2.1. Materials

Chemicals such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4',6-diamidino-2-phenylindole (DAPI), acridine orange (AO), 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), JC-1 and EX-527 were purchased from Sigma (München, Germany). Amyloid- β (A β) 1–42 was purchased from AnaSpec Inc. (San Jose, CA, USA). We purchased α -Synuclein (SNCA, GeneID: 6622) coding sequence from transOMIC (Huntsville, AL, USA). We purchased antibodies against antibodies against α Syn (#sc-7011-R), AMPK (#sc-25792), p-AMPK (#sc-33524), mTOR (#sc-8319), p-mTOR (#sc-101738), LAMP1 (#sc-17768), caspase 3 (#sc-7148), and poly(ADP-ribose) polymerase (PARP) (#sc-7150) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and antibodies against SOD1 (#GTX13498), FoxO3a (#EP1949Y) and Sirt1 (#E104) were purchased from GeneTex (Irvine, CA, USA). Antibodies against Atg7 (#8558) and Atg12-Atg5 (#4180) were purchased from Cell Signaling Technology (Danvers, MA, USA). The β -actin (#NB600-501) and LC3 (#NB100-2220) antibody was obtained from Novus Biologicals. (Littleton, CO, USA). Primary antibodies were used at a dilution of 1:1000 in 0.1% Tween 20 and secondary antibodies were used at 1:5000 dilutions. All the chemicals were prepared by dissolving phosphate buffer saline solutions stored at -20°C until needed for use in experiments.

2.2. Vector construction and transfection

In α Syn overexpression studies, the human wild-type α Syn coding sequence was amplified and cloned into the SPORT6-pCMV or recombinant adeno-associated viral (rAAV) vectors. For in vitro transfection, cells were transiently transfected with the SPORT6-pCMV-SNCA or control SPORT6-pCMV vector by using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) with Opti-MEM (Gibco, Carlsbad, CA, USA). Cells are typically harvested 24 h post-transfection for studies designed for exposure to A β . For in vivo transfection, rats were stereotaxically injected by adeno-associated viral rAAV-pCMV-SNCA or control rAAV-pCMV vector (viral titer: 1.5×10^{12} genome copies per mL) (Aldrin-Kirk et al., 2014).

2.3. Cell culture and viability assay

Human neuroblastoma SK-N-MC cells were obtained from the American Type Culture Collection (Bethesda, MD, USA). Cells were maintained in Minimal Eagle's medium (MEM; Gibco), supplemented with 10% fetal calf serum, 100 units/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 2 mM L-glutamine at 37°C , 5% CO_2 . The A β solutions were prepared as described previously (Ono and Yamada, 2006). Briefly, A β_{1-42} lyophilizates were dissolved at 10 mM in 10% 60 mM NaOH and 90% 10 mM phosphate buffer (pH 7.4) as a stock reagent, and stored at -78°C until use. For the aggregation protocols, the A β_{1-42} peptide was dissolved in dimethyl sulfoxide to a concentration of 5 mM. Based on the amyloid-derived diffusible ligand protocols, MEM culture medium was added to bring the peptide to a final concentration of 100 μM and incubated at 4°C for 24 h for oligomeric conditions, whereas 10 mM HCl was added to bring the peptide to a final concentration of 100 μM and incubated for 24 h at 37°C for fibrillar conditions (Dahlgren et al., 2002). For viability assay, cells were seeded in 96-well plates at a density of 1×10^4 cells/well overnight and then treated as indicated. After 24 h, the tetrazolium salt MTT was added to the medium following the manufacturer's instructions. Only viable cells could metabolize MTT into a purple formazan product, of which the color density (OD) was further quantified by an EZ Read 400 microplate reader (Biochrom, Holliston, MA, USA) at 550 nm. Cell viability was determined by the percentage of OD of the treated cells divided by that of the untreated controls. For trypan blue dye exclusion assay, the number of viable cells was counted by 0.4% trypan blue stain using a hemocytometer. The result was expressed as a percentage relative to control groups.

2.4. Western blot analysis

After treatment, cells were harvested and homogenized in a protein extraction lysis buffer (50 mM Tris–HCl, pH 8.0; 5 mM EDTA; 150 mM NaCl; 0.5% Nonidet P-40; 0.5 mM phenylmethylsulfonyl fluoride; and 0.5 mM dithiothreitol), and centrifuged at 12,000 g for 30 min at 4°C . The supernatants were used as cell extracts for immunoblotting analysis. To determine the SDS soluble and insoluble fractions of α Syn, samples were homogenized in RIPA buffer (Tris buffer containing 1% Triton X-100; 0.1% SDS; 2 mM MgCl_2 and 0.5% deoxycholate). The solubilized pellet was centrifuged at 15,000 g at 4°C for 30 min, and the supernatant was termed the SDS-soluble fraction and the final pellet the SDS-insoluble fraction (Ihara et al., 2007). Equal protein amounts of total cell lysates were resolved by 10% SDS-PAGE, transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA), and then probed with a primary antibody followed by a secondary antibody conjugated with horseradish peroxidase. The immunocomplexes were visualized with enhanced chemiluminescence kits (Millipore). The relative expression of proteins was quantified densitometrically by using the software Quantity One (BioRad, Hercules, CA, USA), and was calculated according to the reference bands of β -actin. Each blot represents at least three independent experiments.

2.5. Microscopic observation and nucleus morphology

Changes in cell nucleus morphology, characteristic of apoptosis, were examined in cells grown on coverslips, using a microscope. The cells were fixed in 4% paraformaldehyde after 24 h of treatment with the indicated compounds. For phase-contrast inverted microscopy, images of cells were captured with no specific staining procedure. For nucleus morphology microscopy, cells were fixed in ice-cold methanol, and incubated for 15 min at room temperature with 1 ng/mL of 4',6-diamidino-2-phenylindole (DAPI) stain, and observed under a fluorescence microscope (DP80/BX53; Olympus; Tokyo, Japan). Apoptosis was quantified by averaging cell counts in five random 400 \times fields. Values were expressed as the percentage of apoptotic cells relative to total number of cells.

2.6. Immunocytochemistry and acridine orange staining

After treatment cells were fixed with 2% buffered paraformaldehyde, permeabilized in 0.25% Triton X-100 (Sigma-Aldrich) for 5 min at 4 °C, and then incubated with anti- α Syn. The slides were then incubated with an FITC labeled second antibody (Santa Cruz) depending on the origin of the primary antibody. For acridine orange staining, cells were treated with the indicated conditions for 24 h, and then stained with 1 μ g/mL acridine orange for 15 min, washed with MEM medium, and examined under the fluorescence microscope (DP80/BX53; Olympus) and Cell Sense, V 1.9 digital imaging software.

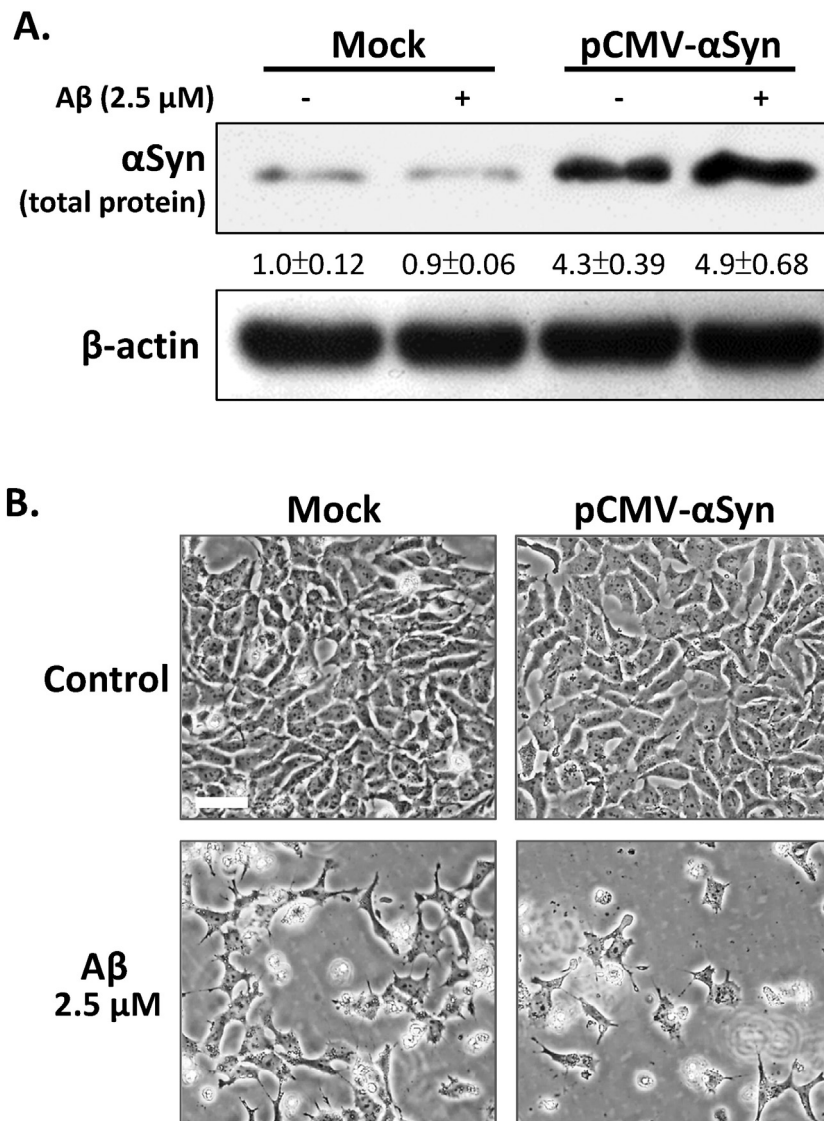


Fig. 1. A β enhances cytotoxicity in α Syn-overexpressed SK-N-MC neuronal cells. (A) SK-N-MC neuronal cells transfected with SPORT6-pCMV-SNCA plasmid show overexpression of wild-type α Syn protein by Western blots. Treatment with 2.5 μ M of A β _{1–42} for 24 h does not significantly alter the α Syn protein levels in α Syn-overexpressed cells. (B) α Syn-overexpressed cells show no morphological evidence of cytotoxicity or apoptosis compared to mock-transduced cells. However, treatment with 2.5 μ M of A β for 24 h markedly induces cell death, and further exacerbation by overexpression of α Syn. (C) MTT assays indicate 2.5 μ M of A β induces cell death by 45% after 24 h incubation, and α Syn overexpression exacerbates A β toxicity up to 72% of cell death. (D) Similarly, trypan blue exclusion assays reveal that A β significantly reduces the number of viable cells compared to mock-transduced groups for 24, 36 and 48 h. (E) 2.5 μ M of monomeric but not oligomeric or fibrillar A β induces synergistic cytotoxic effects in α Syn-overexpressed cells after 24 h incubation. (F) Western blot results demonstrate that A β enhances both caspase 3 and PARP activation in α Syn-overexpressed cells. (G) A β markedly increases the number of apoptotic cells with fragmented nuclei in α Syn-overexpressed cells. Apoptosis is determined by fragmented morphology in the nucleus for DAPI fluorescence. The numbers of apoptotic cells are quantified by averaging cell counts in five random 400 \times fields. Other data were performed in three independent experiments, and values are presented as mean \pm SEM. Significant difference is determined by using the multiple comparisons of Dunnett's post-hoc test for * P < 0.05 and ** P < 0.01 compared to α Syn-overexpressed only groups. Scale bar represents 50 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

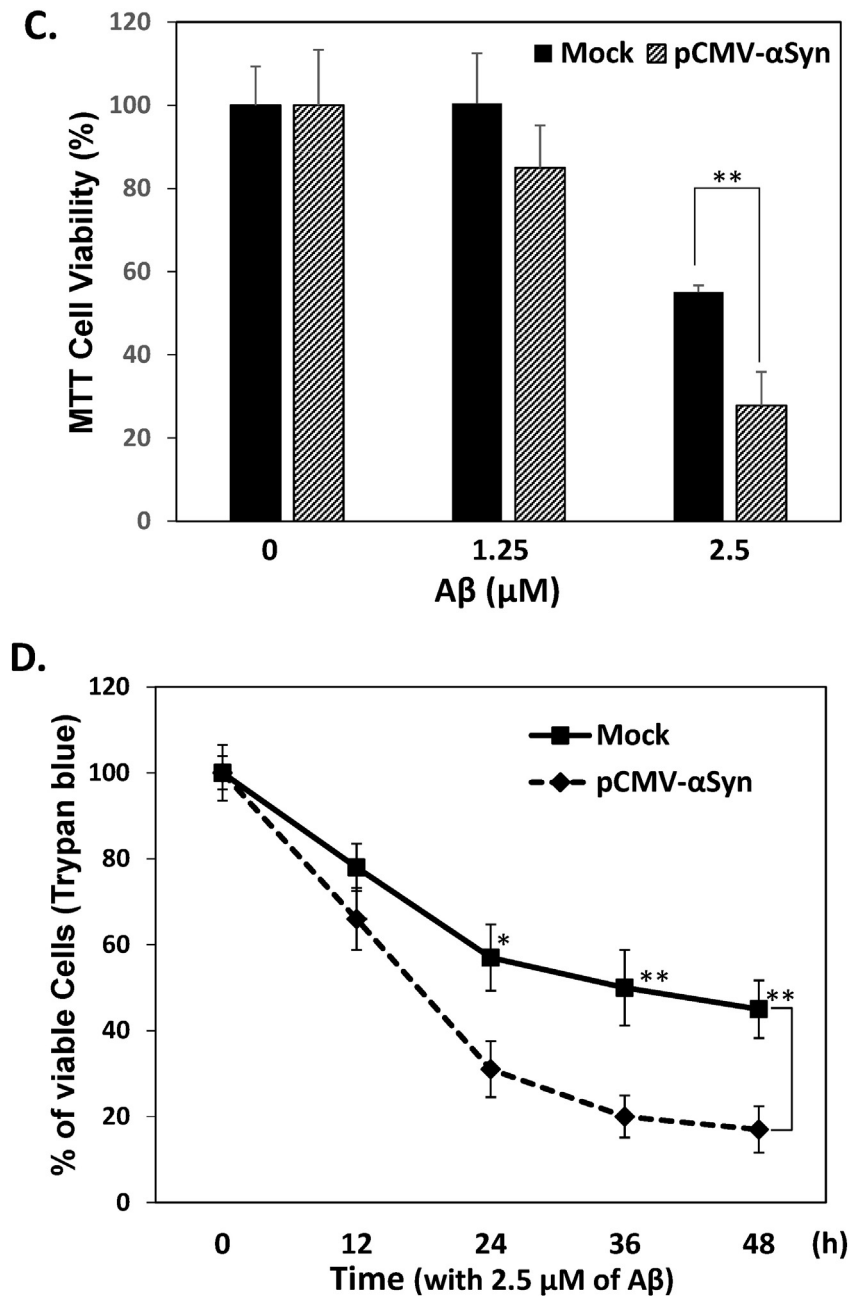


Fig. 1 (continued).

2.7. Analysis of mitochondrial membrane potential

The vital mitochondrial cationic dye JC-1, which exhibits potential-dependent accumulation in mitochondria, was used to investigate mitochondrial function. Cells were treated in fresh medium containing 1 μM JC-1 and were incubated at 37 °C for 30 min. The staining medium was then discarded and the cells were washed. Cells were then imaged using an inverted fluorescence microscope (DP72/CKX41; Olympus) excited at 488 nm. In normal cells, JC-1 continues to exist as aggregates and produces a red fluorescence (~590 nm). During the induction of apoptosis, the mitochondrial potential collapses and JC-1 forms a monomer producing green fluorescence (~525 nm).

2.8. Measurement of reactive oxygen species (ROS)

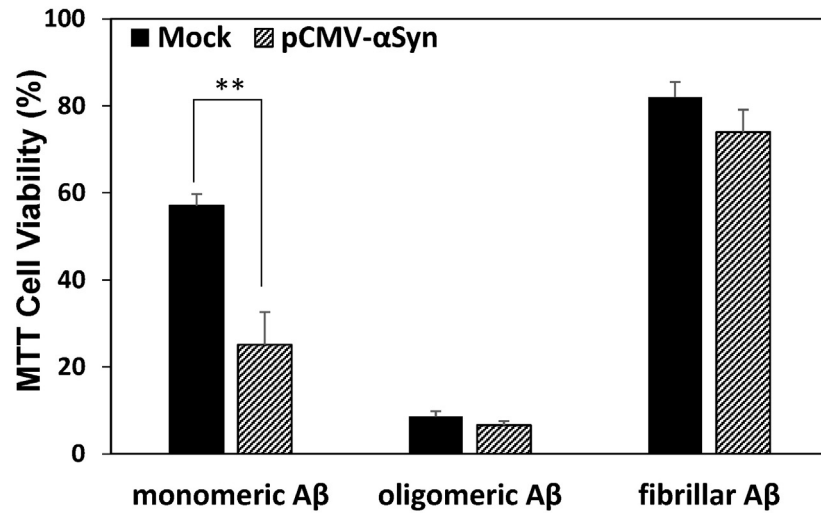
To evaluate the levels of intracellular ROS, cells were seeded onto glass coverslips and incubated with 10 μM of 2',7'-

dichlorodihydrofluorescein diacetate (DCFH-DA, a general oxidative stress indicator) for 0.5 h at 37 °C under 5% CO₂ after treatment. After incubation, the staining medium was discarded and cells were washed twice immediately with PBS, after which the intensity of fluorescence was imaged by fluorescence microscopy (DP72/CKX41; Olympus) using an excitation wavelength of 488 nm and an emission wavelength of 525 nm. One representative image of three different experiments is shown.

2.9. Animal research

Three-month old Wistar rats were kept on a 12-h light/dark cycle and housed individually with free access to food and water. The rats were randomly divided into three groups including control rAAV-pCMV vector, rAAV-pCMV-SNCA vector expressing human wild-type αSyn, and Aβ_{1–42} co-administration in bilateral hippocampus with rAAV-pCMV-SNCA vector by stereotaxic intracerebroventricular (ICV)

E.



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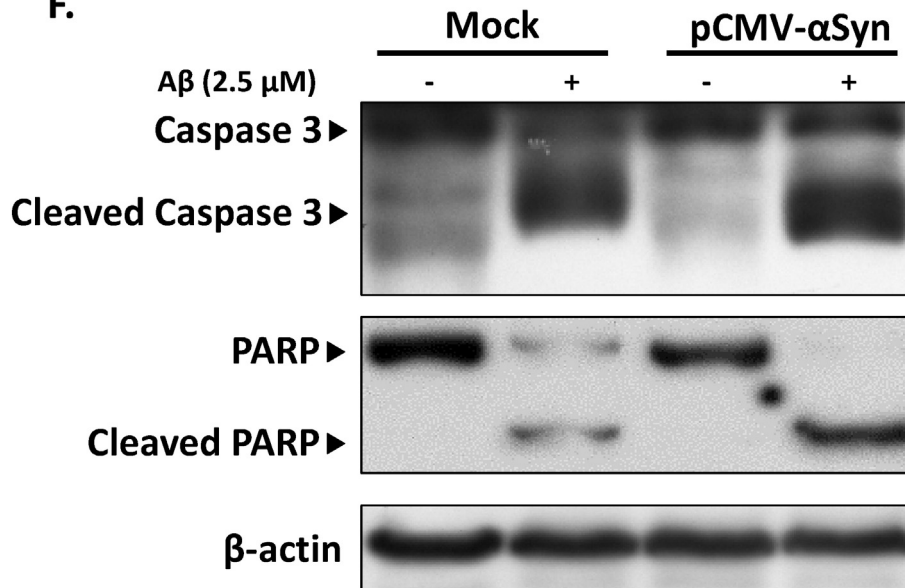


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injection. There were 6 rats for each group, and therefore, a total of 18 rats were used in the current study. All experimental procedures involving animals were approved by the Institute Animal Care and Use Committee (CSMU No. 1394). The ICV injection protocol was in reference to Hsieh et al. (2012). Briefly, rats were anesthetized with Zoletil 50® (10 mg/kg, i.p.; Vibac Laboratories, Carros, France), and mounted in a stereotaxic frame. The rAAV vector solution of 10 μ L was injected into the left lateral cerebral ventricle with the following coordinates, 0.8 mm posterior to the bregma, 1.5 mm lateral from the midline, and 3.8 mm ventral from the skull. The A β_{1-42} solution of 5 μ L (2 μ g/ μ L, 10 μ g each side) was injected into each side of the hippocampus by using the following stereotaxic coordinates: 3.6 mm posterior to the bregma, 2.4 mm left/right to the midline, and 2.8 mm ventral to the bregma. All the injections were performed within 5 min, and following the needle remained in the target location for 10 min to avoid reflux along the needle tract. After four weeks conducting ICV injection, the animals were sacrificed, and the brains were then dissected, and homogenized or fixed immediately. Sagittal sections of 40 μ m thickness

from brain hemisphere were made using a freezing microtome (Leica, Nussloch, Germany).

2.10. mRNA expression analysis by reverse-transcription quantitative PCR (qPCR)

After treatment, total mRNA was extracted by utilizing the kit Qiagen RNeasy Kit (Qiagen, Germantown, MD, USA) and was quantified spectrophotometrically following the manufacturer's instructions. RNReverse transcription was performed at 25 °C for 10 min for primer binding, 37 °C for 120 min for reverse transcriptase and 85 °C for reverse transcriptase denaturation using the TProfessional Thermocycler (Biometra, Göttingen, Germany). Real time quantitative qPCR, using an ABI 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), was performed for quantification of mRNA. PCR amplifications of target mRNA genes were carried out in conjunction with Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. Each cDNA sample was tested in

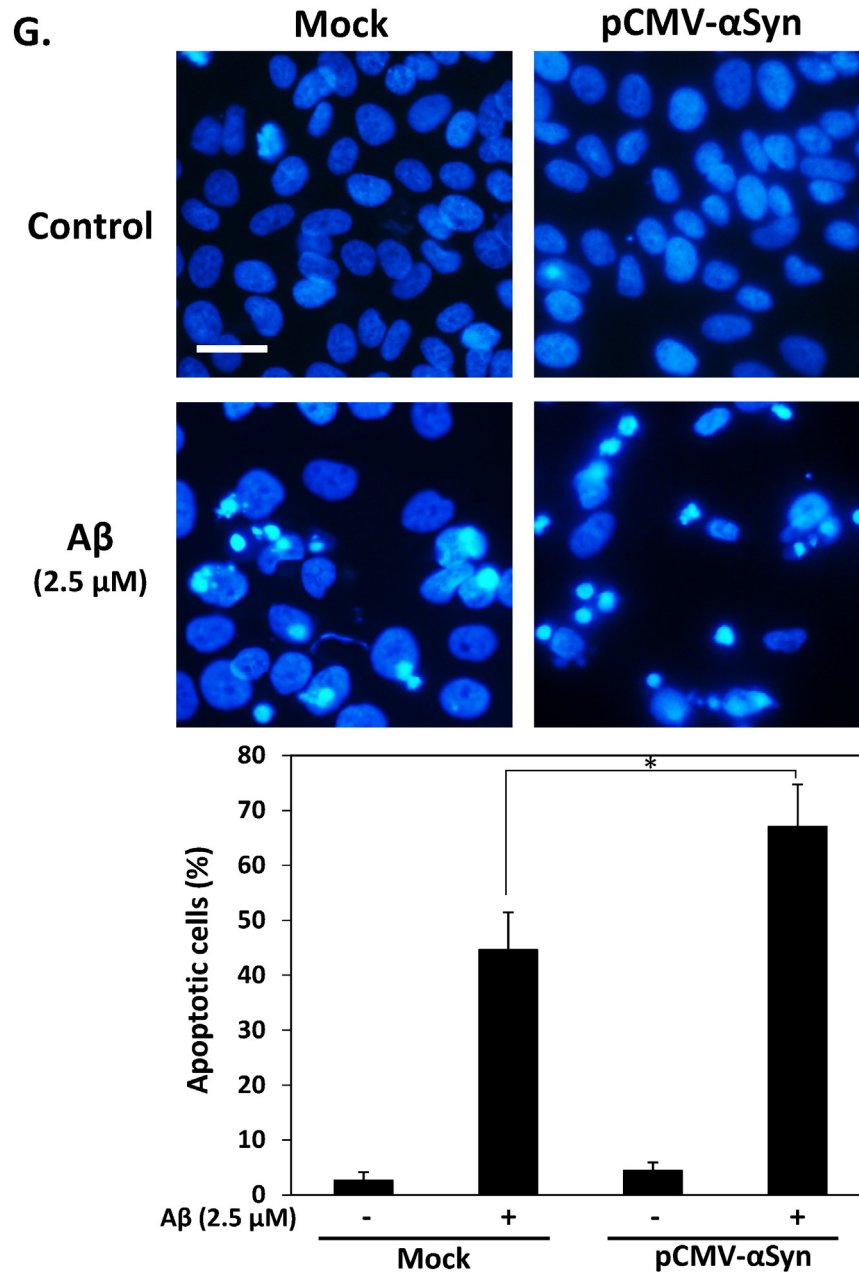


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triplicate. The temperature parameters were the following: 95 °C/10 min, 40 cycles of 95 °C/15 s, 60 °C/1 min and dissociation stage was 95 °C/15 s, 60 °C/15 s and 95 °C/15 s. The following primer pairs were used: forward 5'-TAGCCTTGTCAGATAAGGAAGGA-3' and reverse 5'-ACAGCTTACAGTCAACTTTGT' for Sirt1, forward 5'-GCGTGGCCTAGC GAGTTATG-3' and reverse 5'-TCCTTCTGCTCGAAATTGATGA-3' for SOD1, and forward 5'-CCTCCTGCACCACCAACT-3' and reverse 5'-GACC TTGCCACAGCCTT-3' for GAPDH. Values of relative mRNA expression were obtained by using the software SDS version 1.2.3 (Sequence Detection Systems 1.2.3-7300 Real Time PCR System; Applied Biosystems) and the values were standardized by comparing with values from relative expression of GAPDH.

2.11. Blood samples from human subjects

A total of 10 Chinese human subjects (five with DLB, and five healthy controls) with negative or clinically diagnosed DLB were recruited from

Chung Shan Medical University Hospital and Bodhi Hospital, Taichung, Taiwan. DLB was diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV) criteria. From each subject, 20 mL of venous peripheral ethylenediamine tetra-acetic acid (EDTA) blood was obtained, and total RNA was isolated by utilizing the kit Qiagen RNeasy Kit (Qiagen, Germantown, MD, USA) in accordance with the manufacturer's instructions. The aforementioned protocol was approved by the Chung Shan Medical University Hospital Institutional Review Board (IRB) protocols (CSMUH No: CS12247), and informed consent was also obtained from all participants according to the Declaration of Helsinki and approved by the IRB.

2.12. Statistical analysis

All data are presented as means \pm standard error of the means (SEM). Statistical analysis of data was performed using analysis of variance (ANOVA), followed by Dunnett's post-hoc test for multiple

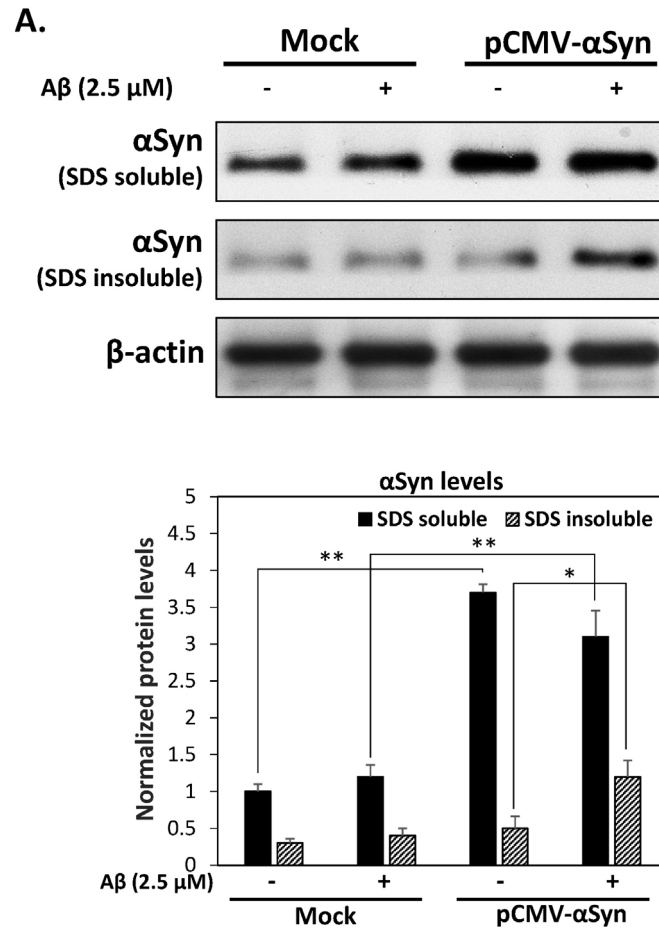


Fig. 2. A β promotes α Syn aggregation and inhibits AMPK-dependent autophagy in α Syn-overexpressed cells. (A) Representative immunoblots and quantitation of α Syn. Proteins soluble or insoluble in 0.1% SDS from lysates of α Syn-overexpressed or mock-transduced cells are analyzed by quantitative blotting with antibodies to α Syn. (B) Immunofluorescence analysis demonstrates that A β significantly increases the number of α Syn aggregates in α Syn-overexpressed cells compared to mock-transduced or non-A β treated groups. (C) Acridine orange staining. Formation of acridine orange-accumulating acidic vacuoles (orange-red fluorescence) in α Syn-overexpressed or mock-transduced cells is significantly suppressed by A β treatment. (D) Western blots show levels of some autophagy-related proteins in both mock and α Syn-overexpressed cells. A β markedly downregulates AMPK Thr¹⁷² phosphorylation and upregulates mTOR Ser²⁴⁴⁸ phosphorylation in α Syn-overexpressed cells. All data were performed in three independent experiments, and values are presented as mean \pm SEM. Significant difference is determined by using the multiple comparisons of Dunnett's post-hoc test for * P < 0.05 and ** P < 0.01. Scale bar represents 20 μ m.

comparisons with SPSS statistical software (SPSS, Inc., Chicago, IL, USA). Differences were considered statistically significant at P < 0.05.

3. Results

3.1. A β significantly enhances cytotoxicity in α Syn-overexpressed SK-N-MC neuronal cells

Although the overexpression of wild-type α Syn has been reported to be nontoxic to neuronal cells, the influence of A β in α Syn-overexpressed cells is not well elucidated. To evaluate whether A β plays a role in α Syn-induced cytotoxicity, we transiently overexpressed wild-type α Syn in SK-N-MC human neuronal cells. Fig. 1A confirmed that α Syn protein levels were about 3-fold greater in overexpressing cells compared to mock-transduced cells by immunoblot analysis. As mentioned above, A β usually correlates with α Syn pathology in DLB patients. To evaluate this cytotoxic effect, after 24 h of α Syn overexpression, 2.5 μ M of A β _{1–42} peptides were added to the culture medium and incubated for an additional 24 h. Our results showed that overexpression of α Syn alone do not induce significant toxicity. Conversely, A β markedly underwent cell death, and this cytotoxic effect was further deteriorated by overexpression of α Syn (Fig. 1B). In accord, MTT assays showed that treatment with A β for 24 h decreased cell viability by 45%. However, overexpression of α Syn exacerbated A β -induced insults, and led to cell death up to 72% (Fig. 1C). The trypan blue exclusion assays

also revealed that A β significantly reduces the number of viable cells compared to mock-transduced groups for 24, 36 and 48 h (Fig. 1D). In addition, Fig. 1E demonstrated that monomers but not oligomers or fibrils of A β show marked synergy with α Syn-mediated cytotoxicity. To precisely determine which mode of cell death is induced by A β in α Syn-overexpressed cells, we examined the expressions of cleaved caspase 3 and poly (ADP-ribose) polymerase (PARP), two typical markers of apoptosis. As shown in Fig. 1F, A β increased cleavage of caspase 3 and PARP, indicating that the apoptosis occurs mainly by A β treatment. However, overexpression of α Syn further enhanced A β -induced cleavage of caspase 3 and PARP in α Syn-overexpressed cells. These results were also confirmed by DAPI nucleus staining that overexpression of α Syn significantly enhances A β -induced nuclei fragmentation as shown in Fig. 1G. Taken together, our results support the idea that A β can enhance apoptosis in wild-type α Syn-overexpressed neuronal cells.

3.2. A β promotes α Syn aggregation and inhibits autophagy in α Syn-overexpressed cells

Because α Syn overexpression significantly increased A β -induced cytotoxicity in neuronal cells, we next examined whether A β can promote α Syn aggregation. As shown in Fig. 2A, both SDS-soluble α Syn levels were significantly increased with or without treatment of A β in α Syn-overexpressed cells. However, A β significantly increased amounts of the SDS-insoluble α Syn in α Syn-overexpressed cells

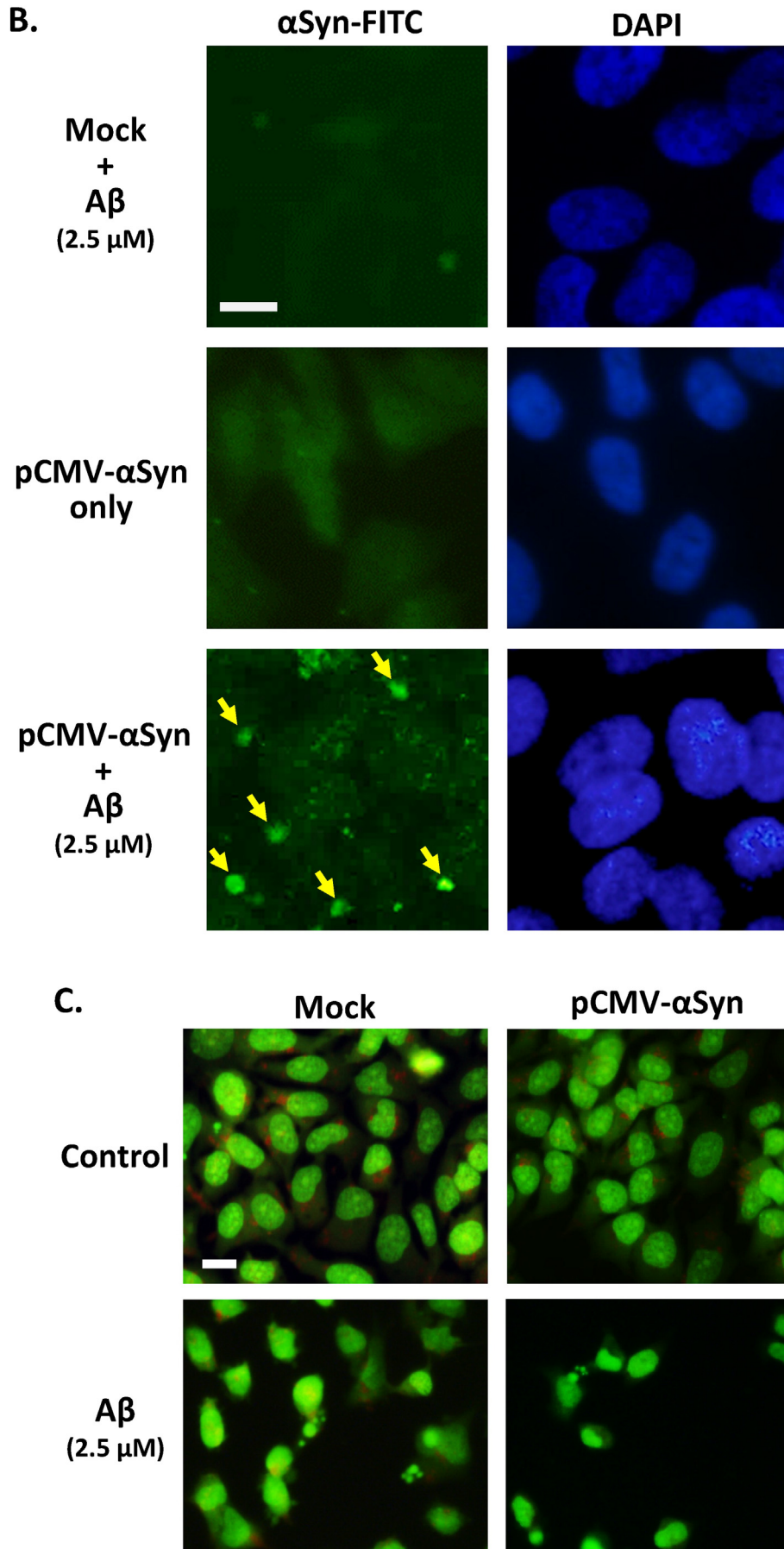


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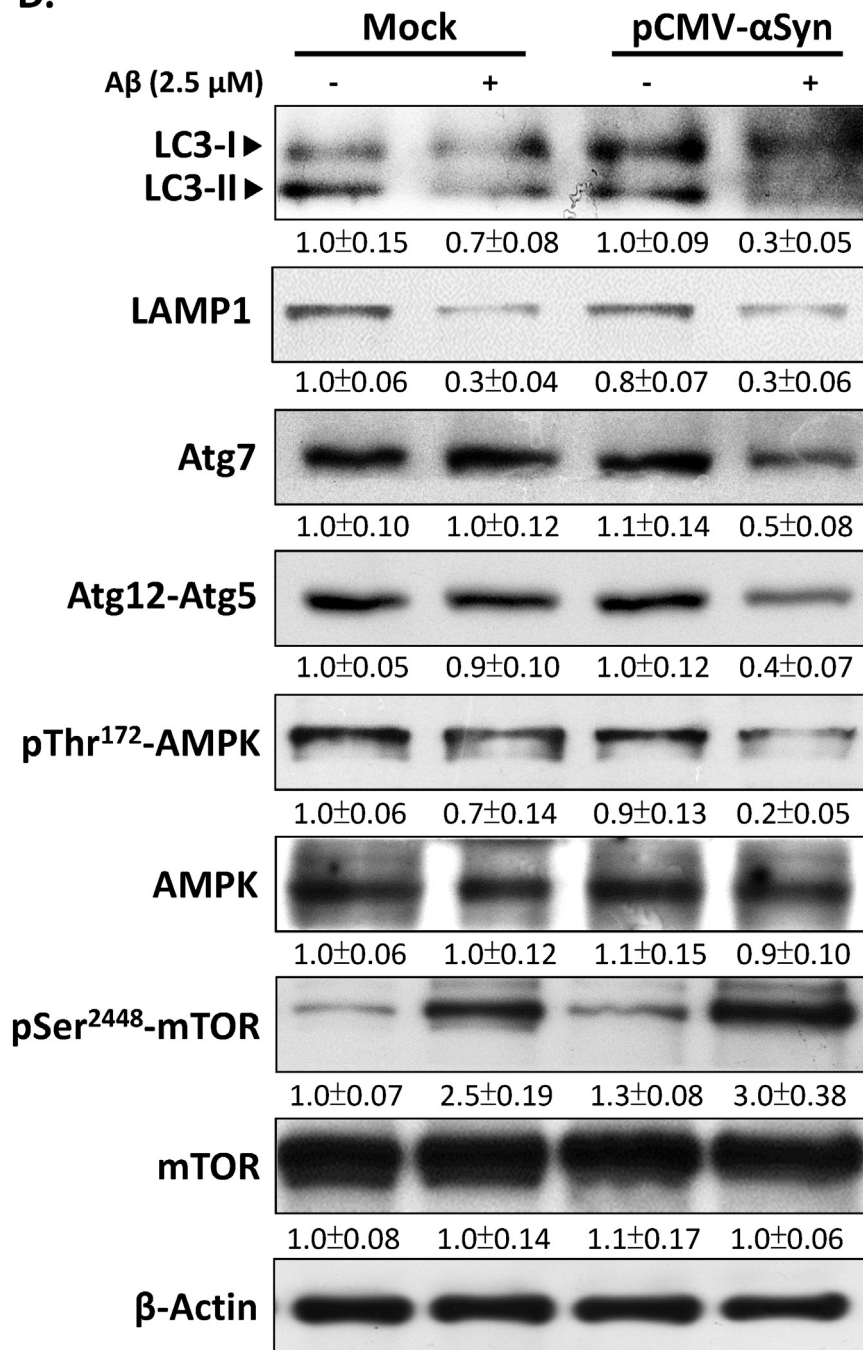


Fig. 2 (continued).

compared to the non-treated group, indicating that the combination of A β and α Syn overexpression may stimulate α Syn aggregation. To further confirm whether A β promotes α Syn aggregation, we performed immunocytochemical staining. As shown in Fig. 2B, mock-transduced cells showed no significant α Syn aggregation. By contrast, overexpression of α Syn significantly increased the number of α Syn aggregates by A β treatment for 24 h, indicating that A β promotes the aggregation of α Syn in α Syn-overexpressed cells. As discussed previously, impairment of the autophagic activity may lead to the accumulation of protein inclusions such as α Syn (Tanaka and Matsuda, 2014). Therefore, we conducted acridine orange staining to examine whether acidic vacuoles (a marker for autophagy) were reduced in α Syn-overexpressed cells by A β . As shown in Fig. 2C, both mock and α Syn transfected cells displayed

detectable orange fluorescence particles by acridine orange staining without A β treatment, indicating the presence of acidic compartments such as autophagolysosome. However, exposure to A β produced a significant decrease in the orange fluorescence, and the overexpression of α Syn further reduced the number of fluorescent spots. These results were also confirmed by Western blotting that A β causes marked inhibition of LC3-II, Atg7, Atg12-Atg5 conjugate, and lysosomal-associated membrane protein 1 (LAMP1) in both vector and α Syn-transfected cells (Fig. 2D). Several lines of evidence have indicated AMPK is a positive regulator in stimulation of autophagy by inhibiting mTOR-dependent signaling. To further elucidate the molecular mechanism of A β -mediated autophagy inhibition, Western blot analysis was conducted to detect the levels of phospho AMPK and mTOR at residue Thr¹⁷²

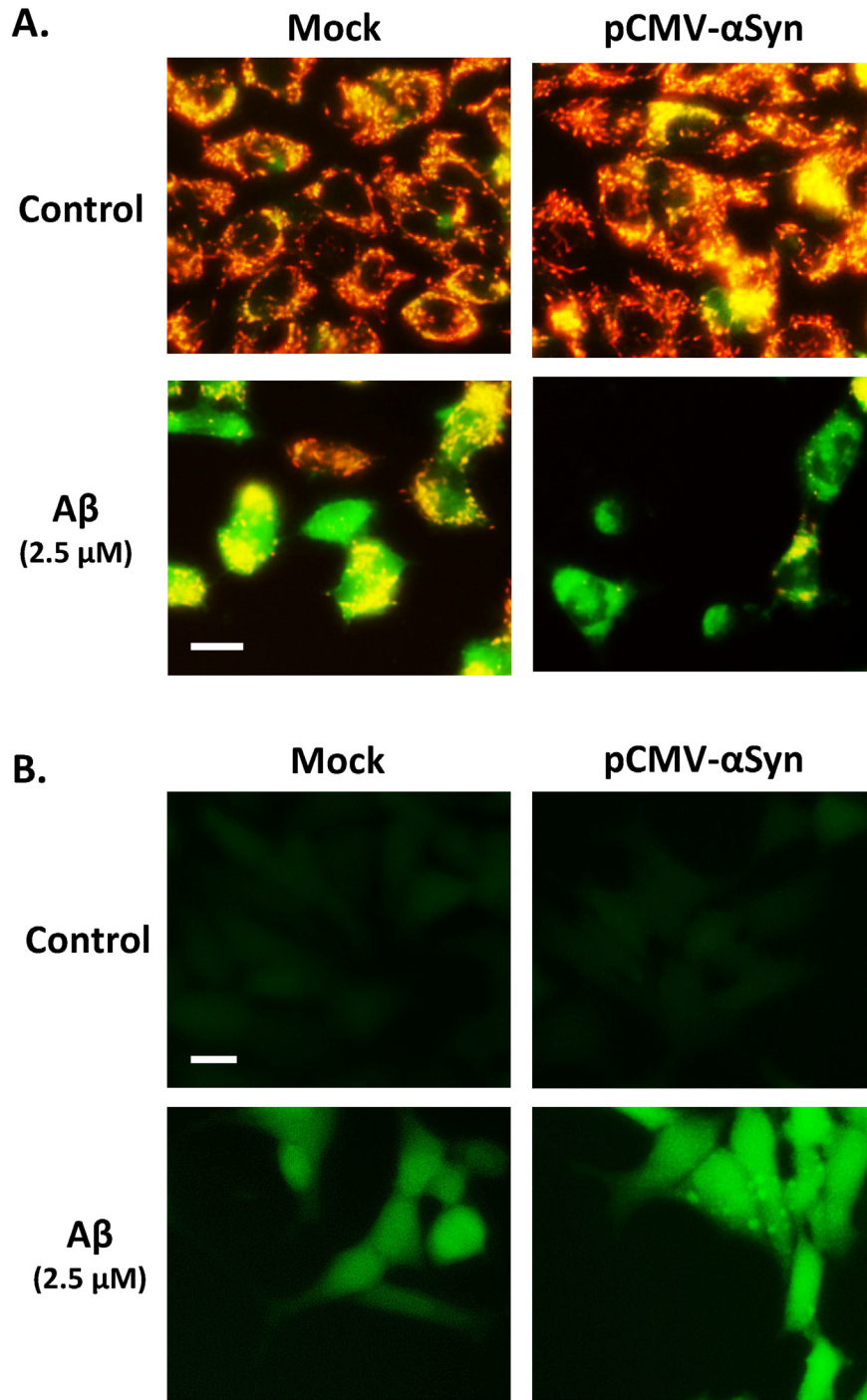


Fig. 3. A β enhances α Syn-induced mitochondrial dysfunction and intracellular ROS accumulation in α Syn-overexpressed cells. (A) JC-1 immunofluorescent staining. Red fluorescence indicates normal mitochondrial membrane potential, and green fluorescence represents A β -enhanced loss of mitochondrial membrane potential in α Syn-overexpressed cells. (B) Effects of A β in enhancing α Syn-induced intracellular ROS accumulation determined by dichlorofluorescein diacetate (DCFH-DA) staining by fluorescence microscopy. (C) Effects of A β on the protein levels of Sirt1, FoxO3a and SOD1 in α Syn-overexpressed cells by immunoblotting. (D) Treatment with 50 μ M of AICAR partially protects against A β -induced cytotoxicity in α Syn-overexpressed cells. However, treatment of cells with 10 μ M of EX-527, a specific inhibitor of Sirt1, significantly abolishes AICAR-mediated protection. All data were performed in three independent experiments, and values are presented as mean \pm SEM. Significant difference is determined by using the multiple comparisons of Dunnett's post-hoc test for * P < 0.05 and ** P < 0.01. N.S., no significant difference. Scale bar represents 20 μ m.

and Ser²⁴⁴⁸, respectively. As shown in Fig. 2D, treatment with A β for 24 h caused a significant increase in Ser²⁴⁴⁸ mTOR phosphorylation, which is recognized as a hallmark of autophagy inhibition. Accordingly, A β also suppressed AMPK Thr¹⁷² phosphorylation, indicating that the suppression of AMPK activity may reduce autophagy by increasing mTOR signaling. Taken together, these data showed that A β -inhibited AMPK-mTOR signaling may lead to a defective autophagic clearance of α Syn aggregates in α Syn-overexpressed cells.

3.3. A β mediates mitochondrial dysfunction and antioxidative stress signaling induced by overexpression of α Syn

Previous studies showed strong evidence that aggregated α Syn can induce mitochondrial deficit and oxidative stress in DLB (Kawamoto et al., 2014; Mullin and Schapira, 2013). To determine whether A β promotes mitochondrial deficit in α Syn-overexpressed cells, we performed JC-1 staining to assess the mitochondrial membrane potential. As

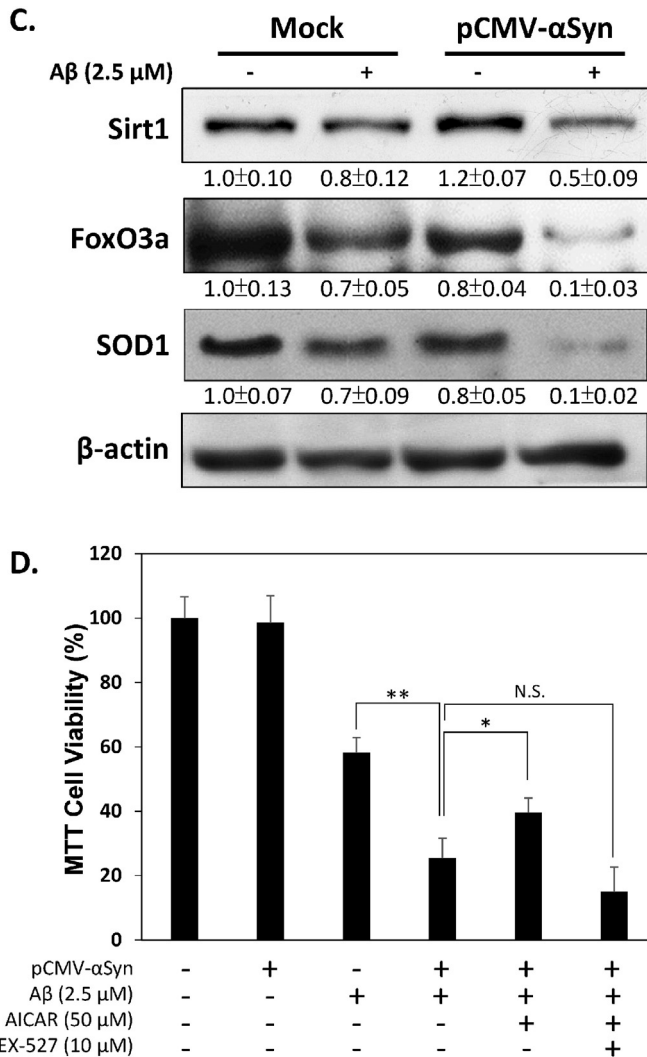


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shown in Fig. 3A, JC-1 aggregates were found in healthy mitochondria by a red fluorescence without Aβ treatment. However, exposure of αSyn-overexpressed cells to Aβ for 24 h resulted in significant increases in green fluorescence, indicating a loss of mitochondrial membrane potential. To gain more insights into mitochondrial oxidative stress, we measured intracellular ROS levels by the 2',7'-dichlorofluorescein diacetate (DCFH-DA) fluorometric method. As expected, our results showed that Aβ significantly increases the burden of intracellular ROS, and this ROS accumulation was further enhanced by overexpression of αSyn (Fig. 3B). This suggests that Aβ enhances ROS accumulation and mitochondrial dysfunction in αSyn-overexpressed cells. It has been demonstrated that Sirt1 can stimulate the expression of ROS detoxification enzymes via FoxO3a signaling (Hasegawa et al., 2008). To test whether such responses involve αSyn aggregation during Aβ-induced oxidative stress, the Sirt1 and FoxO3a protein levels were determined by immunoblotting. As shown in Fig. 3C, Aβ significantly downregulated Sirt1, FoxO3a, and SOD1 levels, suggesting that αSyn-induced cytotoxicity may be enhanced by Aβ-inhibited ROS-scavenging enzymes. This observation was further confirmed by MTT assays. As shown in Fig. 3D, treatment with the AMPK activator 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) can partially protect against Aβ-induced cytotoxicity in αSyn-overexpressed cells, suggesting that the activation of AMPK improves cell viability. By contrast, co-treatment with the Sirt1 inhibitor EX-527 significantly abolished AICAR-mediated protection, indicating that the upregulation of AMPK/Sirt1

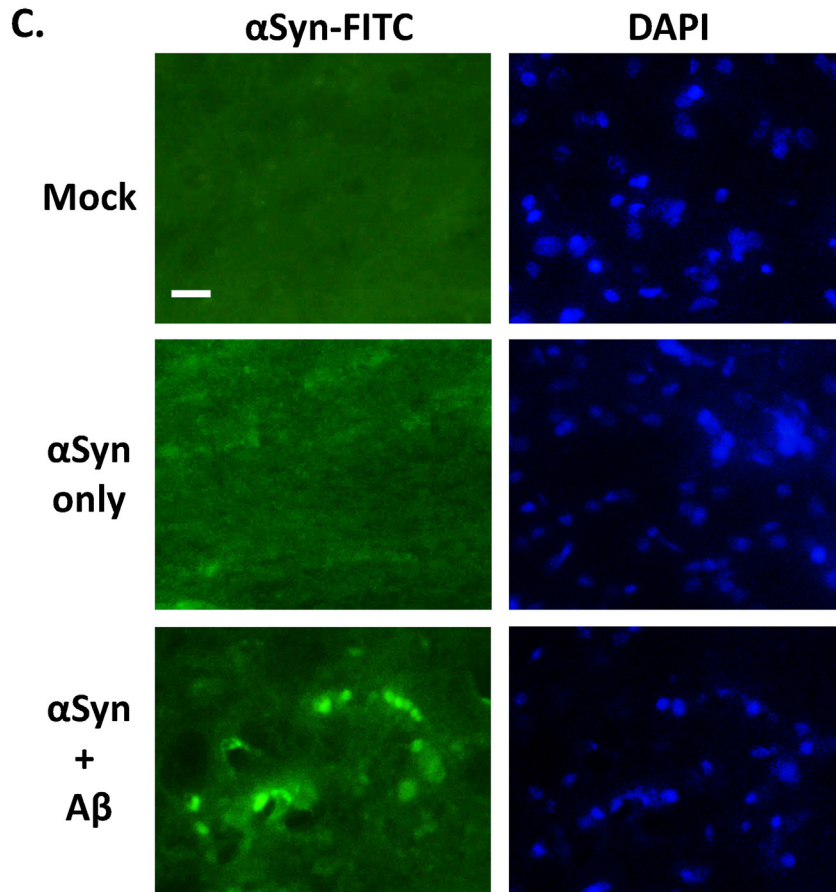
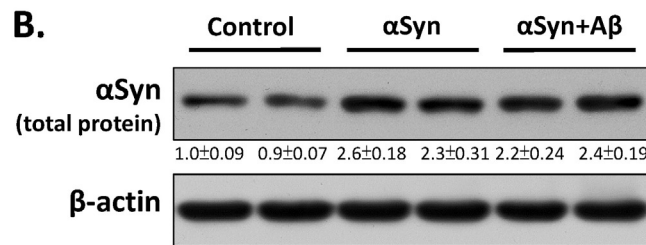
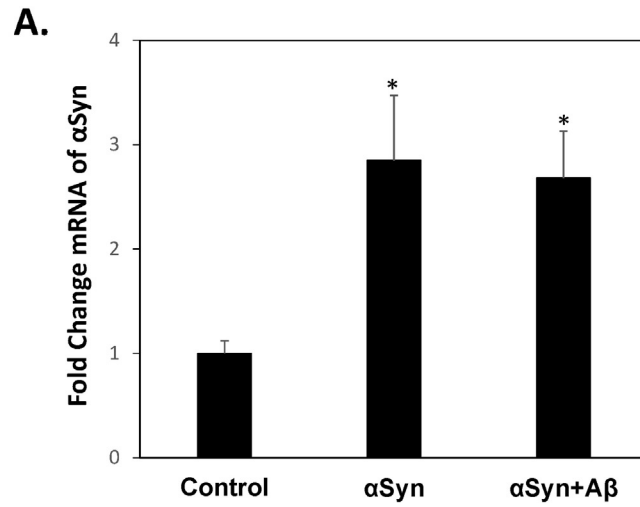
pathway may play a central role in protection against oxidative damage in Aβ and αSyn-induced cytotoxicity.

3.4. Aβ stimulates αSyn aggregation in the αSyn-overexpressed rat hippocampus

Although transformed neuronal-like cell lines can be used to elucidate the toxicological mechanism of αSyn, some doubts still exist when utilizing cells derived from cancerous origin in our present experiments. Therefore, there is a need for animal models to examine more closely DLB pathogenesis in order to mimic an in vivo situation. To create a rat model exhibiting αSyn overexpression in the brain, we delivered human αSyn in a rAAV-pCMV-SNCA into brain ventricular space in mimicking a primarily cerebral type of DLB by stereotaxic surgery (Aldrin-Kirk et al., 2014). In addition, human Aβ₁₋₄₂ peptides were also stereotaxically injected bilaterally into the hippocampus to imitate the action of Aβ. 4 weeks after stereotaxic injection, rats were sacrificed and the brains were dissected and collected immediately. To evaluate the mRNA and protein levels of αSyn, the hippocampus was homogenized and evaluated by qPCR and Western blotting. As shown in Fig. 4A, qPCR analysis revealed that injection αSyn expressing vector in rats significantly increases αSyn mRNA levels (2 to 3-fold) in the hippocampus. Western blot assays also revealed similar results, confirming that hippocampal αSyn is indeed overexpressed by stereotaxic injection (Fig. 4B). Next, we examined whether exposure of rat hippocampal tissues to Aβ contributes to αSyn aggregation. As shown in Fig. 4C, imaging by fluorescence microscopy revealed that no significant aggregated αSyn is found in αSyn-overexpressed hippocampal areas. However, exposure of αSyn-overexpressed hippocampal tissues to Aβ significantly increased the number of αSyn aggregates, indicating that Aβ promotes αSyn aggregation in vivo. To further evaluate these findings, we performed Western blotting to detect the expression of some autophagy-associated proteins in hippocampal tissue lysates. As shown in Fig. 4D, Aβ exposure significantly suppressed AMPK Thr¹⁷² phosphorylation and LC3-II formation in αSyn-overexpressed hippocampal tissues. This was accompanied by the upregulation of mTOR Ser²⁴⁴⁸ phosphorylation, indicating that Aβ may repress autophagy through AMPK/mTOR signaling pathway in vivo. In addition, Aβ also downregulated the mRNA levels of Sirt1 and its related downstream target SOD1 by using qPCR assays in αSyn-overexpressed hippocampal tissues (Fig. 4E). Taken together, these data were in accord with our previous in vitro results, suggesting that Aβ promotes αSyn aggregation in αSyn-overexpressed cells both in vitro and in vivo.

3.5. Peripheral blood leukocyte Sirt1 and SOD1 mRNA levels are reduced in DLB patients

Sirt1 is thought to play an important role in protection against oxidative damage. A previous study has reported that the Sirt1 and its downstream targets are significantly reduced in plasma samples obtained from AD patients (Furuya et al., 2012). Since most AD patients develop Aβ deposits, this raises the possibility that Aβ may also down regulate Sirt1 and its downstream targets SOD1 in DLB patients. However, it remains unclear whether Sirt1 or SOD1 is reduced in DLB patients compared to healthy controls. To evaluate Sirt1 and SOD1 inhibition in DLB patients, peripheral leukocyte mRNAs were isolated and determined in ten human subjects (five healthy controls, five clinically diagnosed DLB patients). A detailed overview of the patient's characteristics is summarized in Table 1. In line with our preliminary expectation, Sirt1 mRNA levels were markedly decreased in DLB patients compared to healthy controls (Fig. 5). Accordingly, the mRNA levels of SOD1 were also significantly reduced in DLB patients. These observations are consistent with our previous results that DLB patients exposed to oxidative stress may be a result of inhibition of Sirt1-related antioxidative pathway, which plays a role in contributing to DLB pathogenesis.



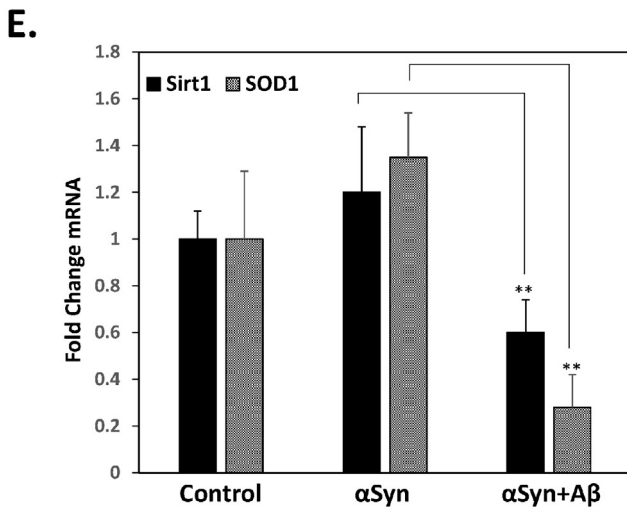
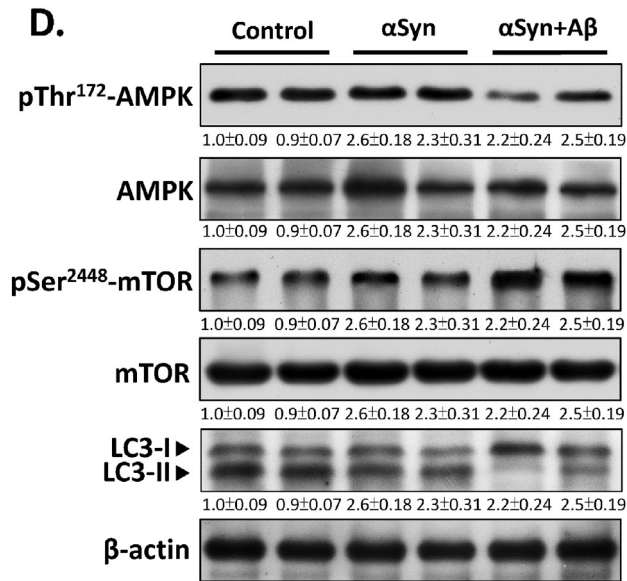


Fig. 4. $A\beta$ increases α Syn aggregates and inhibits AMPK signaling in α Syn-overexpressed rat hippocampus. (A) qPCR analysis reveals that cerebral ventricle injection of rAAV-pCMV-SNCA induces significant mRNA expression in rat hippocampus compared to non-overexpressed control groups. (B) Western blots show that the levels of α Syn protein are also increased in α Syn-overexpressed rat hippocampus. (C) Immunofluorescence images show that exposure of α Syn-overexpressed hippocampal tissues to $A\beta$ increases the number of α Syn aggregates. 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei can be seen in the images on the right column. (D) Determination of the protein levels of p-AMPK/AMPK, p-mTOR/mTOR and LC3-I/II in rat hippocampal tissues by Western blot. (E) qPCR analysis of Sirt1 and SOD1 mRNA levels in rat hippocampal tissues. Both levels of Sirt1 and SOD1 mRNA are significantly reduced in α Syn-overexpressed hippocampal tissues by co-administration of $A\beta$. All data were obtained from three independent experiments, and values are presented as mean \pm SEM. Significant difference is determined by using the multiple comparisons of Dunnett's post-hoc test for * $P < 0.05$ and ** $P < 0.01$. Scale bar represents 100 μ m.

Table 1
Background characteristics of normal controls and patients with DLB.

Characteristics	DLB (n = 5)	Control (n = 5)
Age, years	75.0 \pm 2.2	75.8 \pm 4.8
Sex, female:male	3:2	3:2
MMSE score	19.8 \pm 3.1	27.1 \pm 2.8

Values shown are means \pm SD. DLB, dementia with Lewy bodies. MMSE, Mini-Mental Status Examination.

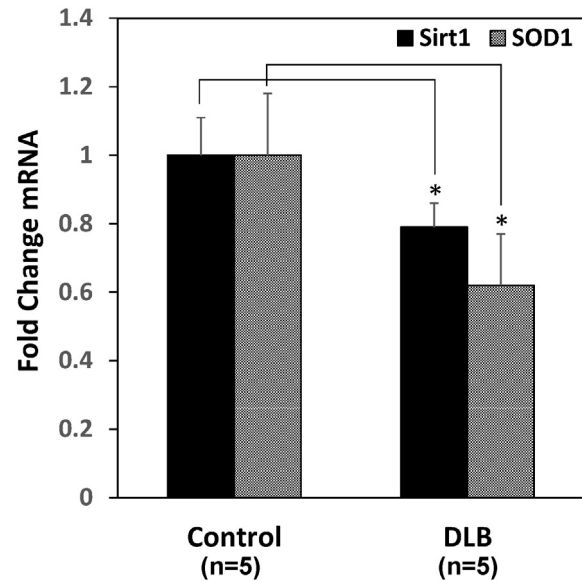


Fig. 5. Peripheral blood leukocyte Sirt1 and SOD1 mRNA levels are reduced in DLB patients. DLB patients show significantly downregulated mRNA expression of Sirt1 and SOD1 in their peripheral blood leukocytes. The mRNA levels of Sirt1 and SOD1 are measured by using qPCR, and results are presented as means \pm SEM. A detailed overview of the patient's background is summarized in Table 1. Statistical analysis is performed by using two-tailed Student's t-test. * $P < 0.05$ when compared with the healthy control groups.

4. Discussion

DLB is considered a synucleinopathy along with PD. However, it shares many features which overlap clinically with both AD and PD. Particularly, DLB is known to demonstrate some AD pathologic features including $A\beta$ deposits and neurofibrillary tangles (McKeith, 2006). In fact, evidence suggests that genes which appear to confer risk of AD will also be of relevance to DLB (Paulson and Igo, 2011). For example, Apolipoprotein E4 allele (ApoE4) is the most common genetic risk factor for AD, which has also been shown to strongly associate with DLB. Conversely, no association is observed between any ApoE alleles and susceptibility to PD or age at onset in PD, indicating that DLB may be more similar to AD than PD in terms of this genetic risk factor (Vefring et al., 2010). As a result, a relationship between $A\beta$ and α Syn-induced neurotoxicity has been suggested by several studies (Marsh and Blurton-Jones, 2012). However, the precise link between these two disease-causing proteins remains unclear. Given the intriguing overlap of DLB and AD pathology, our present study provides a probability of association between $A\beta$ and α Syn-induced neurotoxicity. We demonstrated that $A\beta$ significantly promotes α Syn aggregation and enhances apoptosis in α Syn-overexpressed cells. In addition, we found that the Thr¹⁷² phosphorylation of AMPK is reduced by exposure of α Syn-overexpressed cells with $A\beta$, and this inhibition was likely associated with α Syn aggregation by inhibiting the autophagy. Interestingly, we demonstrated that Sirt1, well-known for longevity signaling, was markedly reduced by $A\beta$ treatment both in vitro and in vivo, indicating that the ablation of Sirt1 and SOD1 expression by $A\beta$ may exacerbate the oxidative stress-induced damage in α Syn-overexpressed cells. Considering that aging is an important and unneglectable factor for the pathogenesis of DLB, our research reveals a potential mechanism that $A\beta$ may enhance α Syn-mediated neurotoxicity by speeding up the aging process. Together, although the precise molecular events underlying α Syn aggregation are still unclear, these results support the idea that $A\beta$ indeed contributes to α Syn aggregation by reducing AMPK signaling in α Syn-overexpressed cells both in vitro and in vivo.

Impairments in the protein degradation in the neuronal cells can lead to an aggregation of toxic proteins, thereby resulting in neuronal dysfunction and neurodegeneration in diseases such as AD and PD

(Fecto et al., 2014). The autophagy process is particularly important for the clearance of toxic protein aggregates in neurons. For example, suppression of autophagy by knocking out autophagy-related genes can lead to severe neurodegeneration (Komatsu et al., 2006). In addition, a previous study has also reported that inhibition of autophagy generates a favorable microenvironment for α Syn aggregation and accumulation (Poehler et al., 2014). In fact, α Syn has been reported to display neuroprotective effects responding to neuronal stress (Aoki and Li, 2011). This indicates that some additional factors or conditions are required for the process of wild-type α Syn aggregation. In accord with these findings, our results demonstrated that simple overexpression of wild-type α Syn in neuronal cells causes little or no obvious neurotoxicity. Notably, Bachhuber et al. have demonstrated that α Syn inhibits rather than promotes A β plaque formation. However, blocking A β fibril formation can increase toxic A β species, thereby leading to a greater neurotoxic effect. In addition, this A β – α Syn hybrid may disrupt membrane integrity and intracellular calcium homeostasis (Bachhuber et al., 2015). Similarly, Wang et al. also indicated that extracellular co-treatment of A β and α Syn can display synergistic neurotoxic effects (Wang et al., 2015). However, it is not clear how these factors interact directly or indirectly in our model. Further studies are needed to explore mechanisms behind such interactions. In addition, increased A β is reported to suppress autophagy via stimulation of mTOR (Godoy et al., 2014), suggesting that A β exposure may exacerbate α Syn-induced neurotoxicity by insufficient autophagic clearance of aggregated α Syn. Considering the important roles of the A β -suppressed AMPK/mTOR signaling in α Syn aggregation and cytotoxicity, our results offer the potential that therapies aimed at enhancing autophagy may represent promising new approaches for treating DLB.

As previously mentioned, α Syn aggregation is thought to induce elevated levels of ROS levels, and is accompanied by progressive mitochondrial dysfunction (Malkus et al., 2009). Interestingly, an abnormally high level of oxidative stress has been found to cause protein misfolding, leading to stimulation of α Syn aggregates and neurodegeneration (Chan et al., 2012). Because A β is known as a major source of oxidative free radicals in the brain (Butterfield et al., 2013), our findings suggest the possibility that A β -induced oxidative stress may also promote α Syn aggregation indirectly. Moreover, our observations also confirmed that Sirt1 is significantly reduced when α Syn-overexpressed cells are exposed to A β . This regulation is in part controlled by FoxO3a, which activates downstream signaling by upregulation of ROS detoxifying enzymes in response to oxidative stress (Hori et al., 2013). Since the Sirt1/FoxO3a axis is known as an evolutionarily well conserved pathway that favors cell survival in the face of oxidative stress (Salminen et al., 2013), the inhibition of Sirt1 signaling by A β can elevate intracellular levels of ROS, which may stimulate α Syn aggregation and render the cells incapable of autophagic clearance. Since A β may serve as a trigger for oxidative stress and protein misfolding (Pimentel et al., 2012), we postulate that A β indeed contributes to α Syn aggregation and deteriorates its neurotoxicity in DLB. To our knowledge, this is the first report demonstrating the AMPK/Sirt1 molecular mechanism of A β in enhancing α Syn aggregation and cytotoxicity. Taken together, our results provide the idea that A β may play a synergistic role in α Syn aggregation and its neurotoxicity. We hope that these new insights into the roles of A β in α Syn pathogenesis will provide a better understanding of treatment or prevention of DLB in future.

Disclosure statement

No actual or potential conflict of interest.

Acknowledgments

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To: tejenlai@hotmail.com

Cc: fmaj7@seed.net.tw, vivid529@hotmail.com, a881612001@gmail.com,
yjho@csmu.edu.tw, katrina82589687@gmail.com, paiyibox@gmail.com,
bathroom79wf@gmail.com, dll@csmu.edu.tw, tejenlai@hotmail.com

11-Oct-2017

Dear Professor Lai,

I am happy to report that the Editor-in-Chief has decided to accept your manuscript entitled "A β exacerbates α -synuclein-induced neurotoxicity through impaired insulin signaling in α -synuclein-overexpressed human SK-N-MC neuronal cells" in its current form for publication in the CNS Neuroscience & Therapeutics.

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A β exacerbates α -synuclein-induced neurotoxicity through impaired insulin signaling in α -synuclein-overexpressed human SK-N-MC neuronal cells

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4 **A β exacerbates α -synuclein-induced neurotoxicity through impaired insulin**
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6 **signaling in α -synuclein-overexpressed human SK-N-MC neuronal cells**
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10 Ching-Chi Chang^{1,2}, Hsin-Hua Li¹, Yen-Ting Chang¹, Ying-Jui Ho³, Ling-Jia Hsieh¹,
11 Pai-Yi Chiu⁴, Yu-Shih Cheng¹, Chih-Li Lin^{1,5} and Te-Jen Lai^{1,2}
12

13
14
15 ¹Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan, ROC.

16
17 ²Department of Psychiatry, Chung Shan Medical University Hospital, Taichung,
18 Taiwan, ROC.

19
20 ³Department of Psychology, Chung Shan Medical University, Taichung, Taiwan,
21 ROC.

22
23 ⁴Department of Neurology, Show Chwan Memorial Hospital, Changhua, Taiwan,
24 ROC.

25
26 ⁵Department of Medical Research, Chung Shan Medical University Hospital,
27 Taichung, Taiwan, ROC.
28
29

30
31
32 Short title: A β exacerbates α -synuclein neurotoxicity
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35
36
37 The first two authors contributed equally to this work
38

39 Corresponding authors

40 Chih-Li Lin, Ph. D. / Associate Professor

41 Institute of Medicine, Chung Shan Medical University.

42 No. 110, Sec. 1, Jianguo N. Rd., Taichung City 40201, Taiwan

43 Phone: +886-4-2473-0022, ext. 11696

44 E-Mail: dll@csmu.edu.tw
45
46

47
48 Te-Jen Lai, M. D./ Ph. D. / Professor

49 Institute of Medicine, Chung Shan Medical University.

50 No. 110, Sec. 1, Jianguo N. Rd., Taichung City 40201, Taiwan

51 Phone: +886-4-2473-9595, ext. 38836

52 E-Mail: ltj3123@ms2.hinet.net
53
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Summary

Aim

α -Synuclein (α Syn) is known as a small soluble protein abundantly expressed in neuronal cells. Although its physiological role is still unclear, the aggregation of α Syn has been recognized as responsible for some neurodegenerative disorders such as dementia with Lewy bodies (DLB). In most cases, intracellular abnormal aggregates are caused by protein-coding mutations that alter primary structure and therefore increase propensity toward aggregation. However, no pathogenic alterations or polymorphisms in α Syn are found in DLB patients so far, suggesting genetic mutations may not play a major role in DLB pathogenesis. In contrast, emerging evidence reveals that amyloid β ($A\beta$) may contribute to aggregate formation and exacerbate neurotoxicity of α Syn. However, the underlying mechanism of action has remained unclear.

Methods

To investigate molecular pathways involved in $A\beta$ -mediated α Syn pathology, we established an *in vitro* model for inducible α Syn overexpression in SK-N-MC human neuronal cells.

Results

Our results demonstrated that $A\beta$ treatment in α Syn-overexpressed neuronal cells significantly increases α Syn intracellular aggregation and cytotoxicity. Moreover, $A\beta$ also caused AMP-activated protein kinase (AMPK) inhibition and impaired insulin sensitivity, which leads to significant downregulation of nuclear factor erythroid 2-related factor 2 (NRF2)/heme oxygenase 1 (HO-1) antioxidant signaling to elicit α Syn aggregation.

Conclusions

This raised the possibility that insulin resistance could be one of the causative factors of α Syn toxicity, and the strategies for insulin sensitization may have therapeutic potential for synucleinopathies including DLB.

Keywords: AMP-activated protein kinase; amyloid β ; dementia with Lewy bodies; insulin resistance; α -synuclein.

Introduction

Dementia with Lewy bodies (DLB) is a form of progressive dementia caused by degeneration of the neurons in the brain. Among adults aged 65 and over, it is widely considered as the common cause of dementia after Alzheimer's disease (AD) and vascular dementia [1]. Clinically, the central features of DLB are composed by progressive decline in both motor and cognitive function, that share symptoms sometimes overlaps with AD or Parkinson's diseases (PD) [2]. At the molecular level, evidence has confirmed that α -synuclein (α Syn), a small (14.4 kDa) protein abundantly expressed in neuronal cells, plays a critical role in the pathogenesis of synucleinopathies such as DLB and PD [3]. Although the exact function of α Syn is not fully known, it is apparently essential for regulation of some neuronal function like synaptic plasticity and development [4]. However, α Syn can also be triggered to generate some cytoplasmic aggregates named as Lewy bodies that are strongly associated with DLB and PD pathology. Actually, aggregated α Syn inclusions are found where neuron death occurs, indicating the aggregation is the main pathogenic cause of α Syn toxicity [5]. Moreover, α Syn aggregates have been demonstrated to alter mitochondrial function and concomitant reactive oxygen species (ROS) intracellular accumulation [6], suggesting oxidative damage may also have an important pathogenic role in DLB [7]. In most cases of neurodegenerative disorders, abnormal aggregates are generally favored by protein-coding mutations that causes conformational changes and results in formation of the misfolded proteins. Although mutations in α Syn can account for a only small fraction of PD cases, no pathogenic alterations or polymorphisms in the *SNCA* gene (encoding α Syn) are found in DLB [8]. In addition, overexpression of wild-type α Syn has been demonstrated non-toxic to human neuronal cells, demonstrating that protein mutations do not play a major role in DLB [9].

Past studies indicate that an overlap of pathologies is found in most individuals with DLB in more than half of AD cases [10]. Similarly, the AD risk factor apolipoprotein E (ApoE) ϵ 4 allele has also been recognized as the strongest genetic risk factor for DLB [11]. These findings further delineate the pathophysiologic connection between AD and DLB. Furthermore, although the accumulation of amyloid β ($A\beta$) peptides in the brain is well known to play a driving mechanism of AD pathogenesis, $A\beta$ plaques are also frequently present in DLB and linked to disease severity [12]. In particular, $A\beta$ (particularly $A\beta_{1-42}$) was shown to effectively promote wild-type α Syn oligomerization *in vitro* [13], and some studies reported $A\beta$ may also

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3 enhance α Syn aggregation and accelerate cognitive decline in animals [14]. These
4 observations indicate that A β may pathologically contribute to the development of
5 DLB by promoting the aggregation of wild-type α Syn. While there is a growing
6 interest in the effects of A β in DLB pathogenesis, the exact role of A β in the DLB
7 pathogenesis and α Syn aggregation are still not fully understood.

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11 Recently, we have reported that exogenous A β can potentially trigger the
12 aggregation and accumulation of wild-type α Syn inside the neuronal cells [15]. By
13 using a transient α Syn-overexpressed model, we demonstrated that A β inhibits
14 autophagy thus causing intracellular accumulation of aggregated α Syn. Nevertheless,
15 A β and α Syn synergistically repressed AMP-activated protein kinase (AMPK)
16 signaling, that ultimately results in oxidative stress-mediated cell death. These results
17 clearly indicated that A β indeed plays an important role in α Syn aggregation and
18 neurotoxicity. However, the *SNCA* gene sequence is not introduced into most cell
19 population during transient expression. Therefore, the effect of target gene
20 overexpression is temporary and unstable, and the impact of transfection-mediated
21 toxicity may also interfere with the data interpretations. To overcome this problem,
22 the present study was undertaken to establish a stable *in vitro* model for inducible
23 overexpression of α Syn in SK-N-MC human neuronal cells. Our results demonstrated
24 that co-treatment of A β in α Syn-overexpressed cells significantly increases α Syn
25 intracellular aggregation and cytotoxicity. This α Syn toxic effect is partly mediated by
26 inactivation of AMPK, which impairs neuronal insulin signaling and leads to
27 downregulation of nuclear factor erythroid 2-related factor 2 (NRF2)/heme oxygenase
28 1 (HO-1) antioxidant pathway. This suggests a simultaneous increase in α Syn and A β
29 may have a synergistic effect for α Syn aggregation and neurotoxicity, which
30 suppresses endogenous antioxidant pathway and causes an increase in oxidative stress
31 due to the impairment of insulin signaling.
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46 **Materials and methods**

47 **Materials**

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49 Chemicals such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
50 (MTT), 4',6-diamidino-2-phenylindole (DAPI), human recombinant insulin, and JC-1
51 were purchased from Sigma (München, Germany). Antibodies against α Syn, AMPK,
52 p-AMPK, mTOR, p-mTOR, cleaved caspase 3, and poly(ADP-ribose) polymerase
53 (PARP) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and
54 antibodies against SOD1, FoxO3a and Sirt1 were purchased from GeneTex (Irvine,
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3 CA, USA). Antibodies against Atg7 and Atg12-Atg5 were purchased from Cell
4 Signaling Technology (Danvers, MA, USA). The β -actin and LC3 antibody was
5 obtained from Novus Biologicals. (Littleton, CO, USA). We purchased α -synuclein
6 (SNCA, GeneID: 6622) coding sequence from transOMIC (Huntsville, AL, USA).
7 Amyloid β (A β) 1-42 was synthesized by LifeTein (Somerset, NJ, USA), and A β
8 solution was prepared as described previously [16]. Primary antibodies were used at a
9 dilution of 1:1000 in 0.1% Tween 20 and secondary antibodies were used at 1:5000
10 dilutions. All the chemicals were prepared by dissolving phosphate buffer saline
11 solutions stored at -20°C until needed for use in experiments.
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19 **Vector construction and transfection**

20 For α Syn overexpression studies, we used a GeneSwitch System (Invitrogen,
21 Carlsbad, CA, USA) to generate SK-N-MC cells inducible expressing human
22 wild-type α Syn by manufacturer's protocols. Briefly, cells were seeded onto 100 mm
23 diameter dishes (1×10^6 cells/dish) in Minimal Eagle's medium (MEM, Gibco)
24 containing 10% FBS 24 h prior to transfection. Human α Syn coding sequence was
25 amplified and cloned into an inducible expression plasmid pGene. For establishment
26 of inducible expression condition, cells were firstly transfected with the regulatory
27 plasmid pSwitch by using Lipofectamine 2000 (Life Technologies), which
28 continuously expresses a mifepristone (MFP)-responsive GAL4 regulatory fusion
29 protein (GAL4-DBD/hPR-LBD/p65-AD). The transfected cells were selected in
30 hygromycin for four weeks in getting stable clones. For α Syn overexpression, these
31 cells were then transfected with a regulatory plasmid pSwitch, and selected in
32 zoecin for additional four weeks in getting another stable clone. Finally, a
33 GeneSwitch-driven SK-N-MC cell line with doubly-stable gene expression,
34 pGene- α Syn/pSwitch, was established. To stimulate α Syn overexpression,
35 GeneSwitch-driven cells were treated with MFP for 48 h, and then co-treated with
36 indicated conditions as described for each experimental design.
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49 **Cell culture and viability assay**

50 Human neuroblastoma SK-N-MC cells were obtained from the American Type
51 Culture Collection (Bethesda, MD, USA). Cells were maintained in minimal Eagle's
52 medium (MEM, Gibco) supplemented with 10% fetal bovine serum, 100 units/mL
53 penicillin, 100 μ g/mL streptomycin, and 2 mM L-glutamine at 37 °C and 5% CO₂.
54 For the cell viability tests, cells were seeded in a 24-well plate overnight and then
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3 treated with the indicated conditions. After 24 h, MTT was added to the medium
4 following the manufacturer's instructions. Only viable cells can metabolize MTT into
5 a purple formazan product, the color density of which was quantified by a Jasco
6 V-700 spectrophotometer (JASCO, Tokyo, Japan) at 550 nm. The average population
7 number of control cells was set to 100% to enable comparison of the survival rates of
8 other tested cells.
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13 14 15 **Western blot analysis**

16 Cells were harvested and homogenized with protein extraction lysis buffer. This
17 buffer contained 50 mM Tris-HCl at pH 8.0, 5 mM ethylenediaminetetraacetic acid,
18 150 mM sodium chloride, 0.5% Nonidet P-40, 0.5 mM dithiothreitol, 1 mM
19 phenylmethylsulfonyl fluoride, 0.15 units/ml aprotinin, 5 µg/ml Leupeptin, 1 µg/ml
20 pepstatin, and 1 mM sodium fluoride. The solution was centrifuged at 12,000 g for 30
21 min at 4 °C to remove debris, and the supernatant cell lysate was used for
22 immunoblotting analysis. Equal amounts (50 µg) of total proteins from the cell lysate
23 were resolved by SDS-PAGE, transferred onto polyvinylidene difluoride membranes
24 (Millipore, Bedford, MA, USA), and then probed with a primary antibody followed
25 by another secondary antibody conjugated with horseradish peroxidase. Primary
26 antibodies were used at a dilution of 1:1000 in 0.1% Tween-20, and secondary
27 antibodies were used at 1:5000 dilutions. The immunocomplexes were visualized
28 using enhanced chemiluminescence kits (Millipore). The relative expression levels of
29 proteins were quantified densitometrically using QuantityOne software (BioRad,
30 Hercules, CA, USA), further normalized according to the housekeeping β-actin
31 protein, and then compared with the normalized protein levels from control cells. The
32 control protein level was then set to 100% for further comparison.
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45 46 **Flow cytometric analysis**

47 At the end of the experiments, cells were harvested by trypsinization. After
48 centrifugation and washed twice, resuspended the cell pellet in PBS and fixed in 70%
49 chilled ethanol. Cells were stained with propidium iodine (PI), and subsequently
50 analyzed for DNA content by using a NovoCyte flow cytometer (NovoCyte 2000,
51 ACEA Biosciences, San Diego, CA, USA), and data analysis was performed by
52 NovoExpress Software. The number of apoptotic cells was quantified by measuring
53 the sub-G1 population
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Immunocytochemistry and acridine orange staining

After treatment, cells were fixed with 2% buffered paraformaldehyde, permeabilized in 0.25% Triton X-100 (Sigma-Aldrich) for 5 min at 4 °C, and then incubated with anti- α Syn primary antibody. The slides were then incubated with an FITC labeled second antibody (Santa Cruz) depending on the origin of the primary antibody. For acridine orange staining, cells were stained with 1 μ g/mL acridine orange for 15 min, washed with MEM medium. Then images were taken using the fluorescence microscope (DP80/BX53, Olympus) and cellSense, V 1.9 digital imaging software.

Microscopic observation and nucleus morphology

Changes in cell nucleus morphology, characteristic of apoptosis, were examined in cells grown on coverslips, using a microscope. The cells were fixed in 4% paraformaldehyde after 24 h of treatment with the indicated compounds. For phase-contrast inverted microscopy, images of cells were captured with no specific staining procedure. For nucleus morphology microscopy, cells were fixed in ice-cold methanol, and incubated for 15 min at room temperature with 1 ng/mL of 4',6-diamidino-2-phenylindole (DAPI) stain, and observed under a fluorescence microscope (DP80/BX53, Olympus). Apoptosis was quantified by averaging cell counts in five random 400 x fields. Values were expressed as the percentage of apoptotic cells relative to total number of cells.

Analysis of mitochondrial membrane potential

The vital mitochondrial cationic dye JC-1 was used as a tool for investigating mitochondrial function which exhibits potential-dependent accumulation in mitochondria. At normal cells, JC-1 continues to exist as a monomer and produces a red fluorescence. During the induction of A β cytotoxicity, the mitochondrial transmembrane potential collapse and JC-1 forms aggregates which produces a red fluorescence. After treatment for indicated conditions, cells were treated in fresh media containing 1 μ M JC-1 and incubated at 37°C for 30 minutes in an incubator. After that staining medium was discarded, washed, and then the cell imaging was performed by using an inverted fluorescence microscope (DP72/CKX41, Olympus).

Statistical analysis

All data are presented as means \pm standard error of the means (SEM). Statistical analysis of data was performed using analysis of variance (ANOVA), followed by

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3 Dunnett's post-hoc test for multiple comparisons with SPSS statistical software (SPSS,
4 Inc., Chicago, IL, USA). Differences were considered statistically significant at * $p <$
5 0.05 and ** $p <$ 0.01.
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8 9 **Results**

10 **Establishing an inducible *in vitro* model for α Syn overexpression in SK-N-MC** 11 **neuronal cells**

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13 To generate a reliable conditional gene expression *in vitro* model, we first used
14 the GeneSwitch System to establish an inducible expression system for α Syn
15 overexpression in SK-N-MC neuronal cells [17]. As shown in Fig. 1A, the
16 GeneSwitch system is based on the binding of a mifepristone (MFP)-responsive
17 GAL4 regulatory fusion protein to GAL4 upstream activating sequences (UAS) in the
18 promoter controlling expression of *SCNA* gene. By co-transfection of two vectors, a
19 regulatory plasmid pSwitch and an inducible expression plasmid pGene- α Syn, cells
20 can be stimulated with MFP to induce α Syn overexpression. To test the induction
21 efficiency for α Syn inducible overexpression, western blot analysis was conducted to
22 detect the expression levels of α Syn at various concentrations of MFP for 24 and 48 h.
23 As shown in Fig. 1B, incubation of GeneSwitch-driven SK-N-MC cells with MFP can
24 function to induce α Syn protein overexpression stimulated by increasing
25 concentrations of inducer MFP at 24 h, and this overexpression was further
26 upregulated at 48 h. In addition, the highest enhancement of α Syn expression was
27 about six-fold higher than that of the control at 10 μ M of MFP at 48 h. Therefore, all
28 subsequent experiments were carried out by using this induction time and MFP
29 concentration to ensure the overexpression of α Syn.
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44 **A β exacerbates α Syn-mediated cytotoxicity in α Syn-overexpressed cells**

45 As reported previously, increased expression of wildtype α Syn seems non-toxic
46 and not to affect neuronal cell response, suggesting some other causal factors such as
47 A β may play a role in α Syn-mediated neurotoxicity. To verify this speculation,
48 GeneSwitch-driven SK-N-MC cells were incubated without or with MFP for 48 h to
49 control the overexpression of α Syn, and then 2.5 μ M of A β ₁₋₄₂ peptides were added to
50 the culture medium for additional 24 h. As shown in Fig. 2A, the phase contrast images
51 revealed that overexpression of wild-type α Syn does not did not show markedly
52 cytotoxicity or morphological changes in cells, indicating α Syn alone is not sufficient
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3 to cell death. A β treatment, on the other hand, resulted in a moderate decrease the
4 viability of non-MFP-induced cells. However, this A β -induced cytotoxic effect was
5 further deteriorated by the overexpression of α Syn. These results were in accordance
6 with the findings obtained from MTT assay, which indicates overexpressed- α Syn
7 significantly enhances A β -induced cell viability loss from $52 \pm 9.6\%$ to $26 \pm 5.7\%$ (Fig.
8 2B). To precisely determine which mode of cell death is induced by A β in
9 α Syn-overexpressed cells, we measured the sub-G1 population by flow cytometry. As
10 shown in Fig. 2C, results revealed that both α Syn-overexpressed cells and control cells
11 show a low sub-G1 fraction without A β treatment. Conversely, A β significantly
12 increased the sub-G1 fraction of α Syn-overexpressed cells compared to non-induction
13 groups, indicating apoptosis may be a major mode of cell death. Further studies in two
14 apoptotic markers cleaved-caspase 3 and cleaved-PARP confirmed that A β -induced
15 apoptosis was synergistically elevated in α Syn-overexpressed cells (Fig. 2D). Taken
16 together, these findings demonstrated that A β synergistically exacerbates
17 overexpressed α Syn-induced apoptosis in SK-N-MC neuronal cells.
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31 **A β exacerbates α Syn intracellular deposits and suppresses AMPK signaling in** 32 **α Syn-overexpressed cells**

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34 It is generally accepted that the formation of α Syn intracellular aggregates is the
35 major pathological hallmark of DLB. To investigate whether exogenous A β treatment
36 stimulates α Syn aggregate formation in α Syn-overexpressed cells, we performed
37 fluorescent immunostaining with a specific antibody against α Syn. As shown in Fig.
38 3A, without A β treatment, no aggregates were observed in cells regardless of α Syn
39 expression levels. On the contrary, only very few aggregates were observed in cells
40 overexpressing α Syn only, but these aggregates were markedly increased in
41 α Syn-overexpressed cells co-incubated with A β . This implicates A β may contribute to
42 the process of intracellular aggregation or accumulation of α Syn. Some studies have
43 also proposed that impaired autophagy function can led to the accumulation of
44 abnormal aggregate-prone proteins inside the cell [18]. To determine whether A β
45 could interfere with autophagy, immunoblotting analysis was performed to detect
46 autophagic activity by evaluating some autophagy-associated markers such as LC3,
47 Atg7 and Atg12-Atg5 conjugate. As shown in Fig. 3B, no obvious LC3-II inhibition
48 was observed in cells without A β treatment. Conversely, LC3-II levels dropped
49 slightly in control cells by A β treatment, and this LC3-II inhibition was further
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3 enhanced by overexpression of α Syn. The same, other autophagy-related markers
4 Atg7 and Atg12-Atg5 conjugate also displayed a similar pattern of reduction in
5 LC3-II levels. It has been reported that AMPK can induce autophagy via inhibition of
6 mTOR, and the inhibition of AMPK by A β has also been observed in neuronal cells
7 [19], suggesting the suppression of AMPK signaling may result in decreased
8 autophagic clearance [20]. As Fig. 3C shown, these observations were confirmed by
9 our results that A β causes markedly activation of mTOR Ser²⁴⁴⁸ phosphorylation,
10 which possibly as a result of the reduction in phosphorylation of AMPK at Thr¹⁷².
11 These results were also confirmed by acridine orange staining that overexpressing
12 α Syn only did not decrease the number of orange fluorescence particles (as markers
13 of autophagosomes) However, A β causes markedly decreases the orange fluorescence
14 particles in α Syn-overexpressed cells (Fig. 3D). Altogether, our data demonstrated
15 A β -suppressed AMPK signaling may cause upregulation of mTOR activity, which
16 leads to reduced clearance of α Syn aggregates in α Syn-overexpressed cells.
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27 **Reduced neuronal insulin signaling activity is observed in α Syn-overexpressed** 28 **neuronal cells co-treated with A β**

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30 Our previous research has shown that A β can trigger neuronal insulin resistance,
31 which may be functionally linked to the neurodegenerative processes [21]. To
32 determine whether the A β -enhanced α Syn neurotoxicity is associated with impaired
33 neuronal insulin signaling, we performed western blotting to detect the level of IRS-1
34 phosphorylation in Ser³⁰⁷, a typical marker indicates the severity of insulin resistance.
35 As shown in Fig. 4A, the serine-phosphorylated IRS-1 exhibited reduced expression
36 levels in cells without A β treatment. On the contrary, A β caused an increased
37 expression of serine-phosphorylated IRS-1, and this increase was further upregulated
38 by overexpression of α Syn, suggesting neuronal insulin signaling is impaired by A β .
39 To elucidate the role of insulin signaling in A β and α Syn-induced cell death, MTT
40 assays were performed to assess cell death in the presence or absence of exogenous
41 insulin. As shown in Fig. 4B, A β significantly enhanced α Syn-induced cell death
42 without adding insulin in culture medium. Conversely, this cell death was attenuated
43 by exogenous supplement of 0.1 μ M or 1 μ M insulin in a dose-dependent manner,
44 demonstrating that upregulation of insulin signaling can protect against A β -induced
45 cell death in α Syn-overexpressed cells. These results were also confirmed by DAPI
46 staining that treatment with insulin significantly reduced nuclei fragmentation as
47 shown in Fig. 4C, indicating A β -caused apoptotic events were attenuated by
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3 co-treatment of insulin in α Syn-overexpressed cells.
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7 **Insulin alleviates A β -induced α Syn aggregation and oxidative stress in**
8 **α Syn-overexpressed cells**
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10 As mentioned earlier, autophagy plays a pivotal role of in the clearance of
11 intracellular misfolded protein such as α Syn aggregates. Hence, to identify the
12 molecular basis of insulin-mediated neuroprotection, western blot analysis of
13 autophagy-related proteins was performed as in Fig. 5A. Unexpectedly, the levels of
14 LC3-II were shown to increase slightly in α Syn-overexpressed cells co-treated with
15 A β and insulin. Similarly, the addition of insulin caused only moderate effects on
16 increased phosphorylation at Thr¹⁷² of AMPK. These results implied that
17 insulin-mediated neuroprotection in α Syn-overexpressed cells would be unlikely
18 through the induction of autophagy. Since the aggregated α Syn is thought to play a
19 central role in DLB pathogenesis, we therefore investigated if A β -induced α Syn
20 aggregation can be attenuated by insulin. As shown in Fig. 5B, our results from
21 immunofluorescence analysis revealed that compared to the A β +MFP group, the
22 supplement of exogenous insulin significantly reduced the number of fluorescent
23 spots, indicating insulin treatment effectively suppresses A β -induced α Syn
24 aggregation in α Syn-overexpressed cells. It has been reported that oxidative stress can
25 stimulate α Syn synuclein oligomerization via ROS increase-driven protein
26 posttranslational modifications [22]. To elucidate whether insulin protects against
27 A β -induced α Syn aggregation is mediated through reduction of oxidative stress, we
28 performed western blot analysis to measure the levels of some antioxidant
29 signaling-related proteins. As shown in Fig. 5C, the levels of Nrf2 and of its target
30 gene HO-1 were markedly reduced by A β in α Syn-overexpressed cells. However,
31 adding insulin to A β -treated cells resulted in upregulation of this antioxidant pathway
32 significantly. The result of Nrf2/HO-1 downstream protein expression also provided
33 further evidence that the levels of ROS detoxifying enzyme SOD1 were effectively
34 restored by insulin treatment. Finally, we performed JC-1 staining to assess the
35 mitochondrial membrane potential to further examine the details of insulin-mediated
36 neuroprotection. As shown in Fig. 5D, exposure of α Syn-overexpressed cells to A β
37 resulted in an increase in green fluorescence, indicating a great loss of mitochondrial
38 membrane potential. However, co-treatment with insulin reduced the effects of A β on
39 mitochondrial membrane potential significantly, suggesting that insulin preserves
40 mitochondrial function against A β -induced α Syn toxicity. Taken together, these results
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3 showed that A β -enhanced α Syn neurotoxicity can be related to impaired neuronal
4 insulin signaling which appears to be a deleterious factor leading to increased
5 propensity of α Syn aggregation.
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8 9 10 **Discussion**

11 At present, there is still no cure for DLB, but symptoms sometimes can be
12 alleviated with medications used for either AD or PD, indicating these diseases may
13 have some shared pathogenic mechanisms. Although the common mechanism
14 underlying these neurodegenerations are not fully understood, some factors have been
15 associated with a higher risk of developing dementia. Particularly, impaired insulin
16 signaling is associated with decreased cognitive ability and the development of
17 dementia [23]. It is conceivable that AD can be mediated by defective insulin
18 signaling in the brain [24]. In fact, the proximal cause of brain insulin resistance
19 appears to be the deposition of A β [25]. Similarly, our recent finding also indicated
20 that A β can directly cause insulin signaling blockade in neuronal cells, and thus
21 contributed to the enhanced oxidative stress leading to neuronal damage [26]. All
22 these findings indicate that A β may play a central role in inhibition of insulin
23 signaling in α Syn-overexpressed cells. Interestingly, a previous study indicates that
24 the presence of insulin resistance in patients with type 2 diabetes displays
25 approximately a 40% increased risk of developing PD [27]. In addition, insulin
26 resistance has been recognized to be associated with increased risk and more severe
27 symptoms of PD dementia [28]. This raises the possibility that A β -caused insulin
28 resistance may enhance the development of α Syn aggregation and its neurotoxicity,
29 which has been recognized as a key mechanism involved in PD and DLB
30 pathogenesis [29]. Given these growing links between insulin resistance and α Syn
31 pathology, it is not surprising that A β may exacerbate α Syn toxicity by disrupting
32 neuronal insulin signaling in α Syn-overexpressed cells, and therefore suggests that the
33 insulin signaling pathway may be a novel therapeutic target for disease treatment.
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48 Our results also demonstrated that A β may suppress Nrf2/HO-1 endogenous
49 antioxidant pathways via inhibition of insulin signaling. Actually, Nrf2 has been
50 found to mediate the expression of a number of antioxidant genes, suggesting
51 increased Nrf2 signaling may be able to suppress oxidative stress in brain tissues [30].
52 Notably, an insulin-sensitizing agent metformin which likely acts directly through
53 activation of AMPK, was reported displaying their neuroprotective potential by
54 increasing the level of Nrf2 and HO-1 in the brain [31]. In parallel with this finding,
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3 our previous study also demonstrated that restoration of A β -induced neuronal insulin
4 resistance can increase Nrf2/HO-1 signaling and subsequently elevate antioxidant
5 enzyme expression [26]. All these supported that attenuated AMPK activation and
6 impaired insulin signaling may act as a detecting factor representing an underlying
7 mechanism of α Syn pathology. On the other hand, evidence suggesting that oxidative
8 stress elicited by mitochondrial dysfunction may be involved in the process of
9 α Syn-induced neurotoxicity [32]. Particularly, *in vitro* studies have shown that
10 increasing levels of oxidative stress can lead to α Syn aggregation [33]. In fact,
11 oxidative stress-induced posttranslational modifications have been demonstrated to
12 play a major role in α Syn oligomerization and toxicity [34]. Moreover, ROS
13 generated by dysfunctional mitochondria appears to accelerate α Syn phosphorylation
14 at Ser¹²⁹ [35], which has been identified as a pathological prognostic marker for
15 synucleinopathy [36]. Based on these findings, we conclude that A β -suppressed
16 insulin signaling may cause inhibition of Nrf2/HO-1 and result in oxidative damage to
17 α Syn, which modulates post-translational modifications and finally affect protein
18 aggregation. However, the precise mechanism of action for α Syn aggregation remains
19 to be fully elucidated.

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21 Recently, Bassil *et al.* reported that the use of glucagon-like peptide-1 analogue
22 exendin-4, an antidiabetic drug improving insulin sensitivity and action, has positive
23 effects on α Syn pathologies of a multiple system atrophy animal model [37]. In
24 accordance with our results, this indicates the restoration of neuronal insulin
25 sensitivity can be a potential therapeutic target for treatment of these Lewy body
26 diseases. These imply that antidiabetic drug acts against insulin resistance may
27 provide some symptom relief for neurodegenerative synucleinopathy including DLB,
28 since there are no effective treatments for these diseases. To our knowledge, this is the
29 first study to shed light on the role and molecular mechanism of insulin signaling on
30 α Syn neurotoxicity. In summary, the current study demonstrated that overexpression
31 of α Syn alone shows nontoxic to SK-N-MC human neuronal cells. However,
32 co-treatment of A β in α Syn-overexpressed cells significantly increased α Syn
33 intracellular aggregation and neurotoxicity. Our results also raised the possibility that
34 A β -induced neuronal insulin resistance could indeed be one of the causative factors of
35 α Syn aggregation, due to suppressed Nrf2/HO-1 antioxidative signaling and
36 mitochondrial dysfunction. However, whether the use of insulin-sensitizing agents for
37 treatment or prevention of α Syn -related neurodegeneration requires further
38 investigation.

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Disclosure Statement

No actual or potential conflict of interest.

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Figure Legends

Figure 1. Establishing an inducible expression system for α Syn overexpression in SK-N-MC cells. (A) Scheme of GeneSwitch System for α Syn inducible overexpression. Incubation of GeneSwitch-driven cells with mifepristone (MFP) can induce α Syn overexpression. (B) Intracellular levels of α Syn were analyzed by western blotting. At 24h post-induction by MFP, there was a slight increase of α Syn levels in a dose-dependent manner, and this overexpression was further upregulated at 48 h. The highest enhancement of α Syn overexpression is about six-fold higher than that of the control at 10 μ M of MFP. All western blotting data were performed in three independent experiments, and the densitometrical values are presented as mean \pm SEM.

Figure 2. Treatment of A β exacerbates α Syn-mediated neuronal apoptosis. (A) MFP-induced α Syn-overexpressed cells showed no morphological evidence of cytotoxicity or apoptosis compared to control cells. However, 2.5 μ M A β markedly induced cell death, and further exacerbated cytotoxicity in α Syn-overexpressed cells. (B) MTT assays indicated A β induces cell death by 48% after 48 h incubation, and α Syn overexpression exacerbated A β -stimulated cytotoxicity up to 74% of cell death. (C) Flow cytometry data analysis using propidium iodide DNA staining demonstrated that A β significantly increased the sub-G1 fraction of α Syn-overexpressed cells compared to non-induction groups. (D) Western blot results showed that A β enhances caspase 3 and PARP cleavages in α Syn-overexpressed cells. All data were performed in three independent experiments, and values are presented as mean \pm SEM. Significant difference is determined by using the multiple comparisons of Dunnett's

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3 post-hoc test for $*P < 0.05$ and $**P < 0.01$. *N. S.*, no significant difference. Scale bar
4 represents 100 μm .
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8 **Figure 3.** A β activates mTOR by inhibiting AMPK and increases αSyn aggregates in
9 αSyn -overexpressed cells. (A) Fluorescent immunostaining images demonstrated that
10 few αSyn aggregates were observed in αSyn -overexpressed only cells. However,
11 these aggregates were markedly increased by co-treating with A β for 24 h. (B)
12 Western blots showed the protein levels of some autophagy-associated markers
13 including LC3-II, Atg7 and Atg12-Atg5 conjugate. A β treatment obviously inhibited
14 the expression of these autophagy-related proteins suggesting reduced autophagy in
15 these cells. (C) Effects of A β on the protein levels of p-AMPK and p-mTOR. The
16 result showed that A β increases Ser²⁴⁴⁸ phosphorylation of mTOR and inhibits Thr¹⁷²
17 phosphorylation of AMPK in αSyn -overexpressed cells. (D) Formation of acridine
18 orange-accumulating acidic vacuoles (orange-red fluorescence) is markedly reduced
19 by A β treatment. Scale bar represents 10 μm .
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29 **Figure 4.** A β impairs neuronal insulin signaling in αSyn -overexpressed cells. (A)
30 Immunoblotting revealed that treatment with A β for 24 h upregulates the
31 phosphorylation of Ser³⁰⁷-IRS-1, and this increase was further upregulated by
32 overexpression of αSyn . (B) MTT assay revealed that without insulin A β significantly
33 enhanced αSyn -induced cell death; however, this cell death was attenuated by
34 exogenous supplement of insulin in a dose-dependent manner. (C) DAPI staining
35 yielded similar results, thereby indicating that treatment with insulin significantly
36 reduced A β -induced nuclei fragmentation in αSyn -overexpressed cells. All data were
37 performed in three independent experiments, and values are presented as mean \pm SEM.
38 Significant difference is determined by using the multiple comparisons of Dunnett's
39 post-hoc test for $*P < 0.05$ and $**P < 0.01$. Scale bar represents 20 μm .
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48 **Figure 5.** Treatment of insulin suppresses A β -induced αSyn aggregation and
49 oxidative stress. (A) Western blotting results revealed that treatment with insulin
50 causes limited effects on increased AMPK Thr¹⁷² phosphorylation and LC3-II levels,
51 suggesting that impaired autophagy activity cannot be fully restored by insulin. (B)
52 Immunofluorescence analysis showed that αSyn aggregates were markedly decreased
53 by treating with insulin in A β -treated αSyn -overexpressed cells. Scale bar represents
54 10 μm . (C) Some antioxidant signaling-related proteins including Nrf2, HO-1 and
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3 SOD1 were analyzed by western blotting, and inhibitory effects of this antioxidant
4 pathway were effectively restored by insulin treatment. (D) JC-1 immunofluorescent
5 staining. Green fluorescence represents A β -induced mitochondrial dysfunction in
6 α Syn-overexpressed cells by dissipation of mitochondrial membrane potential. Red
7 fluorescence indicates that co-treatment with insulin preserves an intact mitochondrial
8 membrane potential. Scale bar represents 20 μ m.
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For Review Only

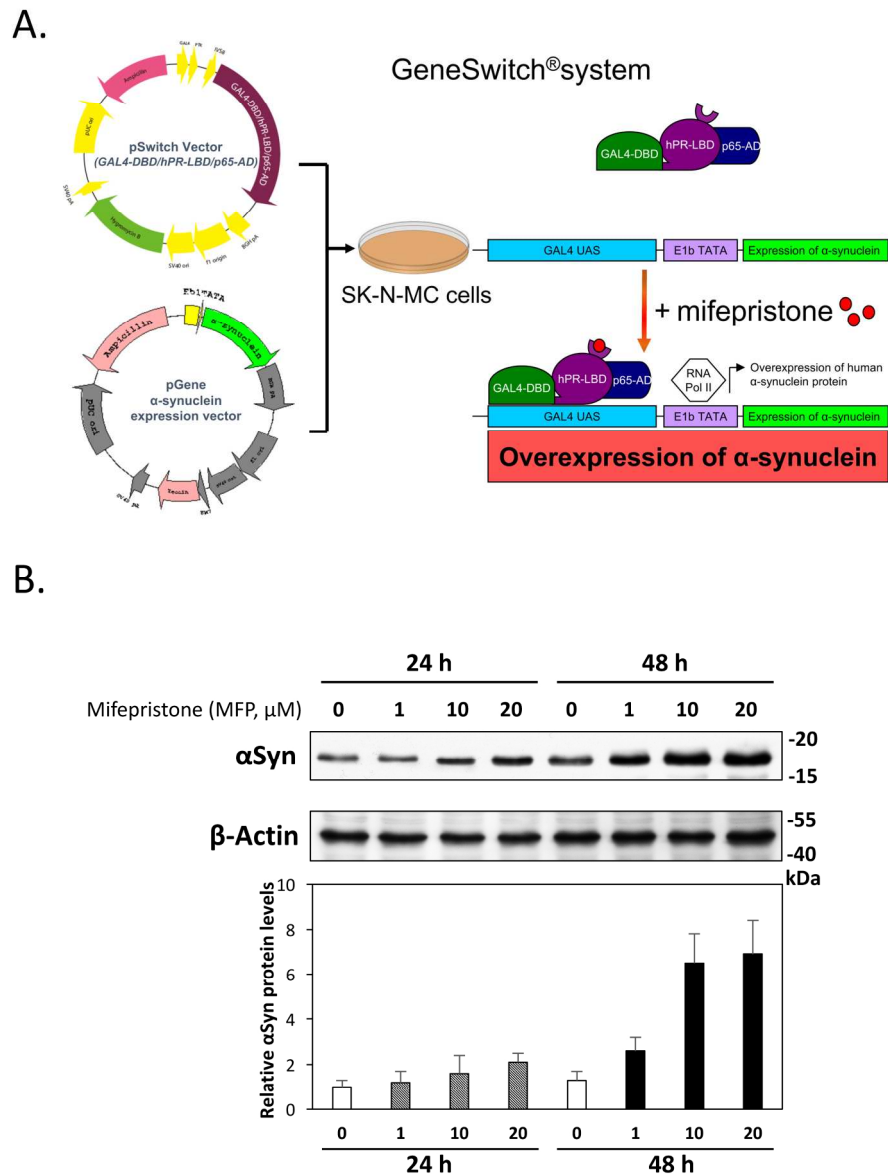


Figure 1. Establishing an inducible expression system for α Syn overexpression in SK-N-MC cells. (A) Scheme of GeneSwitch System for α Syn inducible overexpression. Incubation of GeneSwitch-driven cells with mifepristone (MFP) can induce α Syn overexpression. (B) Intracellular levels of α Syn were analyzed by western blotting. At 24h post-induction by MFP, there was a slight increase of α Syn levels in a dose-dependent manner, and this overexpression was further upregulated at 48 h. The highest enhancement of α Syn overexpression is about six-fold higher than that of the control at 10 μ M of MFP. All western blotting data were performed in three independent experiments, and the densitometrical values are presented as mean \pm SEM.

177x227mm (300 x 300 DPI)

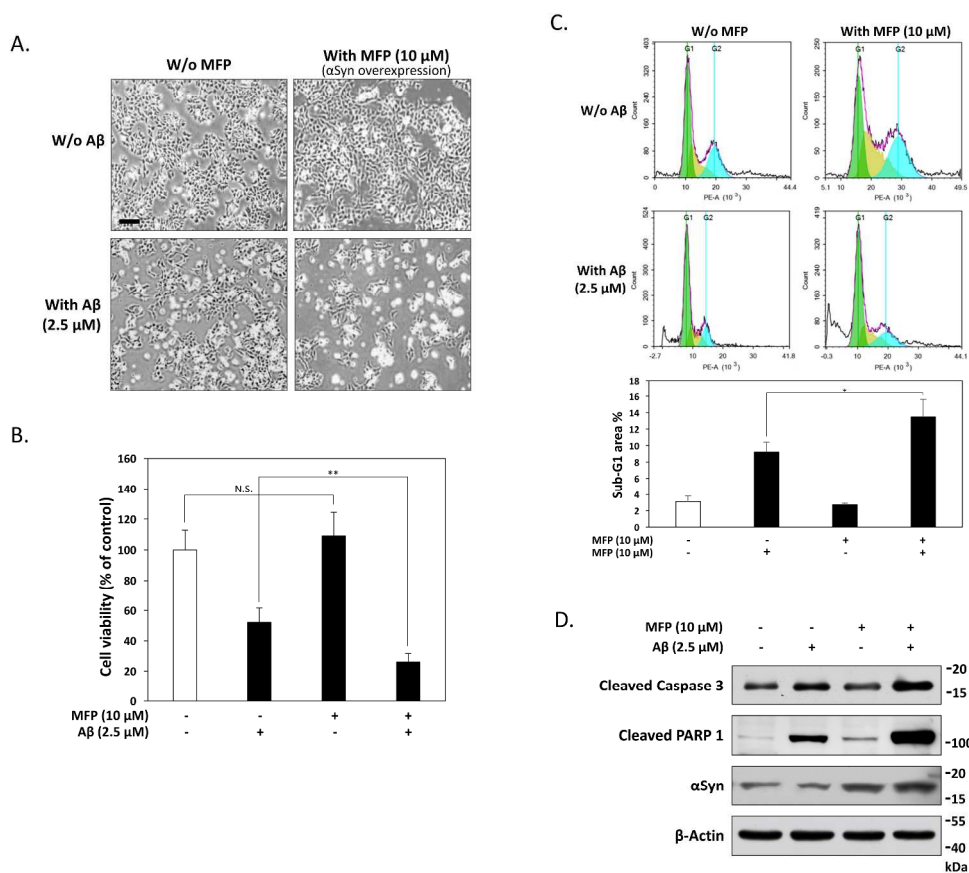


Figure 2. Treatment of Aβ exacerbates αSyn-mediated neuronal apoptosis. (A) MFP-induced αSyn-overexpressed cells showed no morphological evidence of cytotoxicity or apoptosis compared to control cells. However, 2.5 μM Aβ markedly induced cell death, and further exacerbated cytotoxicity in αSyn-overexpressed cells. (B) MTT assays indicated Aβ induces cell death by 48% after 48 h incubation, and αSyn overexpression exacerbated Aβ-stimulated cytotoxicity up to 74% of cell death. (C) Flow cytometry data analysis using propidium iodide DNA staining demonstrated that Aβ significantly increased the sub-G1 fraction of αSyn-overexpressed cells compared to non-induction groups. (D) Western blot results showed that Aβ enhances caspase 3 and PARP cleavages in αSyn-overexpressed cells. All data were performed in three independent experiments, and values are presented as mean ± SEM. Significant difference is determined by using the multiple comparisons of Dunnett's post-hoc test for *P < 0.05 and **P < 0.01. N.S., no significant difference. Scale bar represents 100 μm.

342x303mm (300 x 300 DPI)

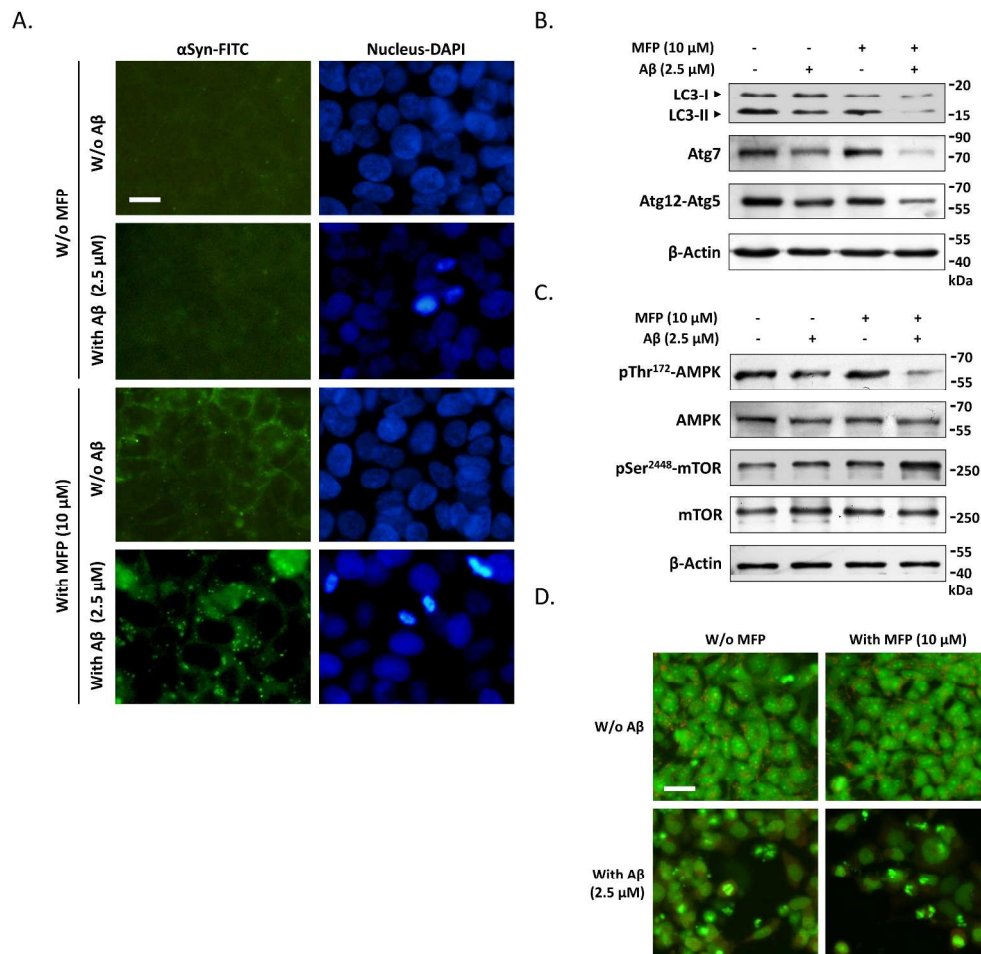


Figure 3. A β activates mTOR by inhibiting AMPK and increases α Syn aggregates in α Syn-overexpressed cells. (A) Fluorescent immunostaining images demonstrated that few α Syn aggregates were observed in α Syn-overexpressed only cells. However, these aggregates were markedly increased by co-treating with A β for 24 h. (B) Western blots showed the protein levels of some autophagy-associated markers including LC3-II, Atg7 and Atg12-Atg5 conjugate. A β treatment obviously inhibited the expression of these autophagy-related proteins suggesting reduced autophagy in these cells. (C) Effects of A β on the protein levels of p-AMPK and p-mTOR. The result showed that A β increases Ser2448 phosphorylation of mTOR and inhibits Thr172 phosphorylation of AMPK in α Syn-overexpressed cells. (D) Formation of acridine orange-accumulating acidic vacuoles (orange-red fluorescence) is markedly reduced by A β treatment. Scale bar represents 10 μ m.

321x313mm (300 x 300 DPI)

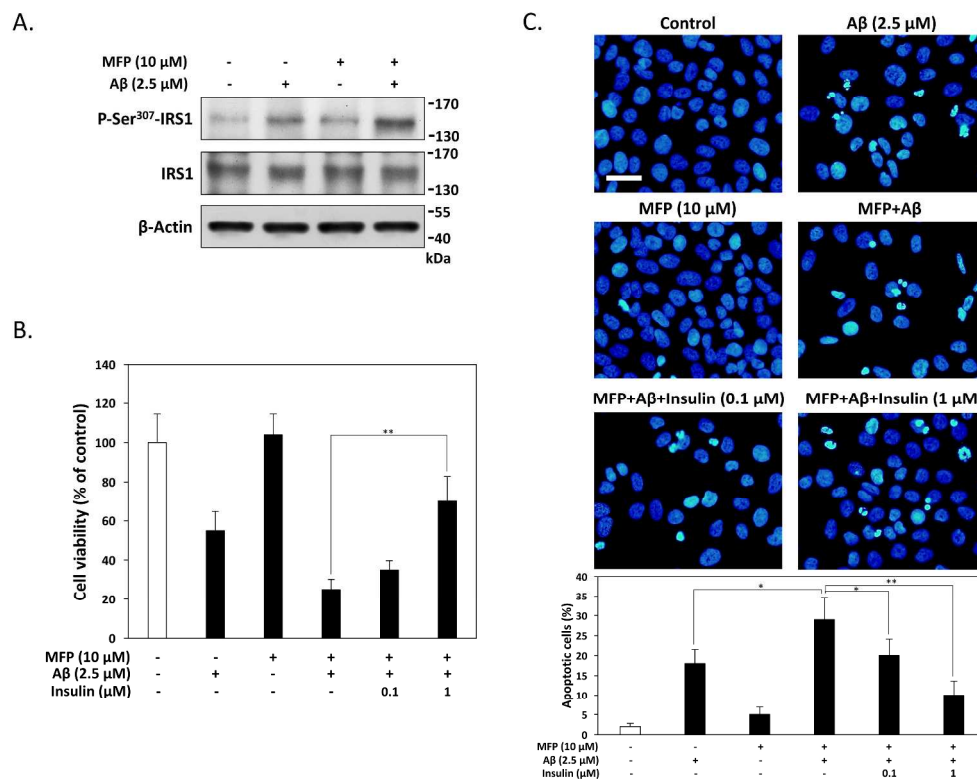


Figure 4. A β impairs neuronal insulin signaling in α Syn-overexpressed cells. (A) Immunoblotting revealed that treatment with A β for 24 h upregulates the phosphorylation of Ser307-IRS-1, and this increase was further upregulated by overexpression of α Syn. (B) MTT assay revealed that without insulin A β significantly enhanced α Syn-induced cell death; however, this cell death was attenuated by exogenous supplement of insulin in a dose-dependent manner. (C) DAPI staining yielded similar results, thereby indicating that treatment with insulin significantly reduced A β -induced nuclei fragmentation in α Syn-overexpressed cells. All data were performed in three independent experiments, and values are presented as mean \pm SEM. Significant difference is determined by using the multiple comparisons of Dunnett's post-hoc test for * P < 0.05 and ** P < 0.01. Scale bar represents 20 μ m.

311x248mm (300 x 300 DPI)

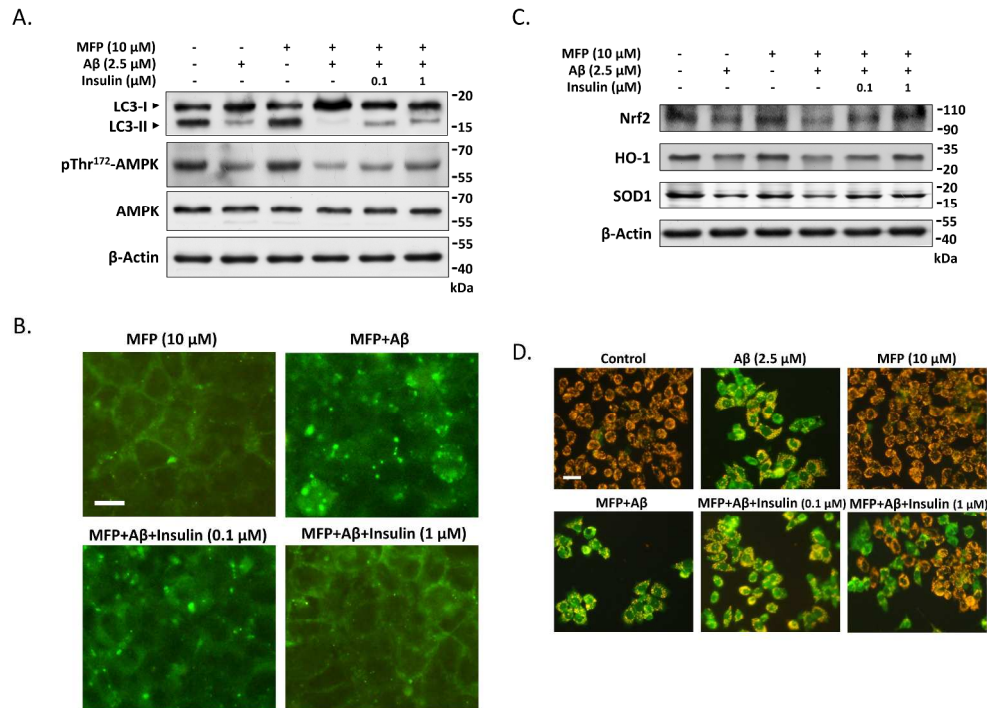


Figure 5. Treatment of insulin suppresses A β -induced α Syn aggregation and oxidative stress. (A) Western blotting results revealed that treatment with insulin causes limited effects on increased AMPK Thr172 phosphorylation and LC3-II levels, suggesting that impaired autophagy activity cannot be fully restored by insulin. (B) Immunofluorescence analysis showed that α Syn aggregates were markedly decreased by treating with insulin in A β -treated α Syn-overexpressed cells. Scale bar represents 10 μ m. (C) Some antioxidant signaling-related proteins including Nrf2, HO-1 and SOD1 were analyzed by western blotting, and inhibitory effects of this antioxidant pathway were effectively restored by insulin treatment. (D) JC-1 immunofluorescent staining. Green fluorescence represents A β -induced mitochondrial dysfunction in α Syn-overexpressed cells by dissipation of mitochondrial membrane potential. Red fluorescence indicates that co-treatment with insulin preserves an intact mitochondrial membrane potential. Scale bar represents 20 μ m.

335x241mm (300 x 300 DPI)

科技部補助專題研究計畫出席國際學術會議心得報告

日期：106 年 9 月 10 日

計畫編號	MOST 104-2314-B-040-007-MY2		
計畫名稱	探討路易氏體失智症之 AMPK-Sirt1 訊息傳遞在 A β 與 α -synuclein 蛋白神經毒性加成效應中可能扮演的角色		
出國人員姓名	賴德仁	服務機構及職稱	中山醫學大學醫學研究所 教授
會議時間	106 年 8 月 7 日至 106 年 8 月 10 日	會議地點	Novosibirsk, Russia
會議名稱	(中文) 2017 年 Belyaev 會議 (英文) Belyaev conference 2017		
發表題目	(中文) 路易氏體失智症中胰島素訊息傳遞調節異常扮演著重要的角色 (英文) Dysregulation of insulin signaling plays an important role in dementia with Lewy bodies (DLB)		

一、參加會議經過

本次會議是由俄羅斯科學院(Russian Academy of Sciences)新西伯利亞分院(Novosibirsk Branch)所舉辦，新西伯利亞分院位於 Novosibirsk 以南約 20 公里的森林中，約有幾十座研究機構在其中，估計從業的專業科學家及相關人員高達數萬人，是全世界知名的高科技科研園區，但全城融合入西伯利亞的白樺森林內，建築物與自然環境相互融合在一起。我們一行人到達當地機場後便由俄羅斯科學院人員驅車接待我們到達會場，本次會議的主要邀請人為 Dr. Tamara G. Amstislavskaya，會議期

間由 8 月 7 日起至 8 月 11 日止共計 4 天，而個人被排定於 8 月 8 日在此會議中發表演講，主題是關於路易氏體失智症在我們研究中的最新進展。路易氏體失智症是僅次於阿茲海默症之老年性失智症，但目前仍無任何已知的有效治療方法，因此亟需尋找新的藥物治療機轉，而我們發現 A β 似乎參與在此症的致病機轉中，同時其病程進展與也 A β 所誘發的胰島素阻抗有關，未來我們也將就這個發現，測試是否能經由給予能改善中樞神經系統胰島素阻抗的藥物，來減緩症狀的惡化速度。在場的人員就此議題的討論相當熱烈，並有多名俄方學者給予我們非常多建設性的建議與回饋，在國際交流方面非常具有實質意義。



中俄雙方部分與會人員在會場合照，我方並致贈俄國友人簡單的紀念品

二、與會心得

本次會議我方受到俄方非常良好且貼心的招待，主辦單位對於遠道而來的我們充分展現了俄羅斯式的熱情與友善。在會議的過程中，中俄雙方均有非常積極的互動及討論，本次會議的主題主要集中在探討神經科學相關的進展，議題包括路易氏

體失智症、阿茲海默症、巴金森氏症、癲癇、憂鬱及思覺失調症等相關疾病的致病機轉及最新的診斷或治療機制等，會後大家的收穫均十分豐碩。比較特別的是俄方特別讓我們參觀了當地的狐狸農場，這個著名的農場是在 1954 年由一位俄羅斯的遺傳學者 Dmitry Belyaev 所創建，其目的是為了探討基因對於馴化過重中的影響力及重要性，本年度的大會紀念主題便是要向 Dr. Belyaev 致敬。此外俄方也帶領我們參觀了他們的附設醫院以及相關研究實驗設施，雙方並根據各自的專業經驗相互分享，希望將來能藉由此發展出國際合作的機會，配合我方的臨床及基礎醫學研究能量，進一步釐清這類疾病的分子醫學機轉，並據此找出未來可能的治療方向。



與 Dr. Amstislavskaya 在 Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences 的代表雕像前合照



參觀當地的附設醫院環境及相關設施

三、發表論文全文或摘要

本次議題以口頭演講的形式進行，發表的照片及摘要如下：



Title: Dysregulation of insulin signaling plays an important role in dementia with Lewy bodies (DLB)

Authors: Te-Jen Lai^{1,2}, Chih-Li Lin¹, Hsin-Hua Li¹, Pai-Yi Chiu³, Ying-Jui Ho⁴

¹Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan.

²Department of Psychiatry, Chung Shan Medical University Hospital, Taichung, Taiwan.

³Department of Neurology, Show Chwan Memorial Hospital, Changhua, Taiwan.

⁴School of Psychology, Chung Shan Medical University, Taichung, Taiwan.

Email: ltj3123@ms2.hinet.net

Abstract

Dementia with Lewy bodies (DLB) is a common cause of geriatric cognitive impairments. The underlying biology of DLB is complex, but the presence of α -synuclein aggregation containing Lewy body is most essential pathological feature. It is interesting many DLB cases are often displayed amyloid β ($A\beta$) depositions, that are crucially involved in Alzheimer's disease (AD). In fact, both α -synuclein and $A\beta$ are frequently co-localized in Lewy body. In addition, the greater the AD pathology in DLB patients, the more severe with disease progression in DLB. However, the reason for this overlap is unknown. As $A\beta$ appears to play a key role in the pathogenesis of DLB, we postulate it might further deepen the progress of α -synuclein-induced neurotoxicity. In the present study, our results demonstrated that $A\beta$ promotes α -synuclein aggregation, and enhances neurotoxicity in a synergistic effect. Moreover, $A\beta$ also decreased the degradation of aggregated α -synuclein by interfering autophagy process, and induced neuronal cell senescence by downregulating of AMP-activated protein kinase (AMPK) and Sirt1 signaling. Interestingly, our data also showed $A\beta$ and α -synuclein-mediated toxic effects are associated with impaired insulin signaling transduction in neuronal cells. Accordingly, restoration of insulin sensitivity by glucagon-like peptide-1 (GLP-1) effectively attenuated $A\beta$ -induced α -synuclein neurotoxicity. In conclusion, our results firstly provided the basis for molecular mechanisms involved in the pathogenesis of DLB and brain insulin resistance. Moreover, the therapeutic potentials of GLP-1 may provide novel treatment strategies for DLB in future.

四、建議

1. 俄方人員在整體大會的流程及周邊生活相關的安排上考慮周到並十分完善，這點對於大多數是第一次到訪俄羅斯的我們非常具有幫助，使遠道而來的我們能在非常短的時間專心投入在大會的活動之中，內心由衷的十分感謝。
2. 此外也要致謝於科技部能支持參加本項極具實質意義的國際會議，相信此次的會議的心得收穫將能有助於我們在未來完成更多更深遠的研究結果。

五、攜回資料名稱及內容

攜回資料共包括：

1. 大會手冊
2. 大會識別證

六、其他



我方人員與俄方學者在大會會場共同合照

104年度專題研究計畫成果彙整表

計畫主持人：賴德仁		計畫編號：104-2314-B-040-007-MY2	
計畫名稱：探討路易氏體失智症之AMPK-Sirt1訊息傳遞在Aβ與α-synuclein蛋白神經毒性加成效應中可能扮演的角色			
成果項目		量化	單位 質化 (說明：各成果項目請附佐證資料或細項說明，如期刊名稱、年份、卷期、起訖頁數、證號...等)
國內	期刊論文	0	
	學術性論文	4	<p>1. Chang YT, Huang WN, Tsai SG, Kim HG, Li HH, Lin YM, Chang CC, Lin CL, Lai TJ*. Aβ promotes αSyn neurotoxicity and cognitive impairment in an αSyn-overexpressed rat animal model. 11th Annual Conference of Taiwanese Society of Geriatric Psychiatry. G12, Kaohsiung, Taiwan, March 2016.</p> <p>2. Chang YT, Tsai SG, Huang WN, Kim HG, Li HH, Lin YM, Lai TJ, Lin CL. Aβ enhances α-Synuclein-induced neuropathology by stereotaxic ICV injections in Wistar rats. 31th Joint Annual Conference of Biomedical Science. P247, Taipei, Taiwan, March 2016.</p> <p>3. Lin CL, Li HH, Chang CC, Ho YJ, Chiu PY, Hsieh CL, Lai TJ*. Investigating molecular mechanisms of Aβ and α-synuclein synergistic effects of neurotoxicity and behavioral deficits in dementia with Lewy Bodies. 2016 International Psychogeriatric Association Asian Regional Meeting. Invited speaker, Taipei, Taiwan, December, 2016.</p> <p>4. Lai TJ*, Hsieh JL, Li HH, Huang WN, Chang CC, Kuo CY, Lin CL. Glucagon-like peptide 1 attenuates dementia with Lewy bodies (DLB) associated neurotoxicity through regulation of insulin resistance. 12th Annual Conference of Taiwanese Society of Geriatric Psychiatry. G15, Taichung, Taiwan, March 2017. (1st class reward)</p>
	專書	0	本
	專書論文	0	章

		技術報告		0	篇	
		其他		0	篇	
智慧財產權 及成果	專利權	發明專利	申請中	1	件	智財局 第1061200908號申請案
			已獲得	0		
		新型/設計專利		0		
	商標權		0			
	營業秘密		0			
	積體電路電路布局權		0			
	著作權		0			
	品種權		0			
	其他		0			
技術移轉	件數		1	件	使用紅血球生成素於治療路易氏體失智症認知功能障礙及神經退化(發明人：何應瑞、賴德仁、林志立)。技轉單位：瑞金生物科技有限公司(2016.02.10)。	
	收入		1000	千元	技轉金額：\$ 1,000,000。	
國外	學術性論文	期刊論文	4	篇	<p>1. Lin CL, Cheng YS, Li HH, Chiu PY, Chang YT, Ho YJ, Lai TJ*. Amyloid-β suppresses AMP-activated protein kinase (AMPK) signaling and contributes to α-synuclein-induced cytotoxicity. <i>Exp. Neurol.</i> 275: 84-98, 2016. (SCI) (IF=4.706, R/C=52/258; 20.2% of NEUROSCIENCES)</p> <p>2. Li HH, Lin SL, Huang CN, Lu FJ, Chiu PY, Huang WN, Lai TJ*, Lin CL. miR-302 attenuates Aβ-induced neurotoxicity through activation of Akt signaling. <i>J. Alzheimers Dis.</i> 50: 1083-1098, 2016. (SCI) (IF=3.731, R/C=77/258; 29.8% of NEUROSCIENCES)</p> <p>3. Chiu PY, Wang CW, Tsai CT, Li HH, Lin CL, Lai TJ*. Depression in dementia with Lewy bodies: A comparison with Alzheimer's disease. <i>PLoS One.</i> 12:e0179399, 2017. (SCI) (IF=2.806, R/C=15/64; 23.4% of MULTIDISCIPLINARY SCIENCES)</p> <p>4. Chang CC, Li HH, Chang YT, Ho YJ, Hsieh JL, Chiu PY, Cheng YS, Lin CL, Lai TJ*. Aβ exacerbates α-synuclein-induced neurotoxicity through impaired insulin signaling in α-synuclein-overexpressed human SK-N-MC neuronal cells. <i>CNS</i></p>	

					Neurosci. Ther. (In press) (SCI) (IF=3.919, R/C=47/256; 18.4% of PHARMACOLOGY & PHARMACY)
		研討會論文		2	1.Lai TJ*, Chang YT, Li HH, Chang CC, Ho YJ, Chiu PY, Lin CL. Synergistic effects of A β on α -synuclein-induced neurotoxicity and behavioral deficits in dementia with Lewy bodies (DLB). 2016 International Psychogeriatric Association International Congress. P36, San Francisco, USA, September 2016. 6.Lin CL, Huang CN, Li HH, Liu GY, Hung HC, Lai TJ*. Roles of ornithine Decarboxylase (ODC) in regulation of amyloid β -induced microglial neuroinflammation. Alzheimer's Association International Conference 2017. P3-05, London, England, July 2017. 2.Lin CL, Li HH, Kim HG, Chang CC, Chiu PY, Ho YJ, Lai TJ*. Co-expression of Oct4, Sox2, Klf4 and Nanog (KOSN) transcription factors protects against amyloid β -induced neurotoxicity by improving insulin signaling sensitivity. Belyaev Conference 2017, Invited speaker, Novosibirsk, Russia, August, 2017.
		專書		0	本
		專書論文		0	章
		技術報告		0	篇
		其他		0	篇
智慧財產權及成果	專利權	發明專利	申請中	0	件
			已獲得	0	
		新型/設計專利	0		
	商標權		0		
	營業秘密		0		
	積體電路電路布局權		0		
	著作權		0		
	品種權		0		
	其他		0		
技術移轉	件數		0	件	
	收入		0	千元	
參 本國籍	大專生		0	人次	

與計畫人力		碩士生	3		張雁婷、蔡善格、謝佳玲
		博士生	1		黃玟儂
		博士後研究員	1		李欣樺
		專任助理	0		
	非本國籍	大專生	0		
		碩士生	0		
		博士生	0		
		博士後研究員	0		
		專任助理	0		
	其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)				

科技部補助專題研究計畫成果自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現（簡要敘述成果是否具有政策應用參考價值及具影響公共利益之重大發現）或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

達成目標

未達成目標（請說明，以100字為限）

實驗失敗

因故實驗中斷

其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形（請於其他欄註明專利及技轉之證號、合約、申請及洽談等詳細資訊）

論文： 已發表 未發表之文稿 撰寫中 無

專利： 已獲得 申請中 無

技轉： 已技轉 洽談中 無

其他：（以200字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性，以500字為限）

DLB在所有退化性失智症中的發生率僅次於AD，但目前仍缺乏有效的藥物來減緩神經元持續的退化，即使給予病人良好的照護與處置，DLB病患的症狀終究不可避免的會持續惡化下去，因此發展機制性的策略將是治療DLB的關鍵。然而DLB的臨床特徵十分複雜，經常合併表現類似AD的失智症表現或類似PD的運動障礙，且在biomarkers方面的檢查包括基因、血液或腦脊髓液等方面也都沒有好的診斷指標。透過執行本計畫，我們已初步釐清DLB的分子致病機轉，特別是證實了A β 在DLB的病程進展中所扮演的角色，並指出其所參與的詳細分子途徑及可能的預防機制，本研究結果將有助於未來開發包括DLB之synucleinopathy相關疾病的預防、診斷與治療策略。

4. 主要發現

本研究具有政策應用參考價值： 否 是，建議提供機關

（勾選「是」者，請列舉建議可提供施政參考之業務主管機關）

本研究具影響公共利益之重大發現： 否 是

說明：（以150字為限）