

Video Article

Visualizing Axonal Growth Cone Collapse and Early Amyloid β Effects in Cultured Mouse Neurons

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URL: <https://www.jove.com/video/58229>

DOI: [doi:10.3791/58229](https://doi.org/10.3791/58229)

Keywords: Neuroscience, Issue 140, Amyloid β , axon, growth cone, collapse, endocytosis, live cell imaging

Date Published: 10/30/2018

Citation: Kuboyama, T. Visualizing Axonal Growth Cone Collapse and Early Amyloid β Effects in Cultured Mouse Neurons. *J. Vis. Exp.* (140), e58229, doi:10.3791/58229 (2018).

Abstract

Amyloid- β (A β) causes memory impairments in Alzheimer's disease (AD). Although therapeutics have been shown to reduce A β levels in the brains of AD patients, these do not improve memory functions. Since A β aggregates in the brain before the appearance of memory impairments, targeting A β may be inefficient for treating AD patients who already exhibit memory deficits. Therefore, downstream signaling due to A β deposition should be blocked before AD development. A β induces axonal degeneration, leading to the disruption of neuronal networks and memory impairments. Although there are many studies on the mechanisms of A β toxicity, the source of A β toxicity remains unknown. To help identify the source, we propose a novel protocol that uses microscopy, gene transfection, and live cell imaging to investigate early changes caused by A β in axonal growth cones of cultured neurons. This protocol revealed that A β induced clathrin-mediated endocytosis in axonal growth cones followed by growth cone collapse, demonstrating that inhibition of endocytosis prevents A β toxicity. This protocol will be useful in studying the early effects of A β and may lead to more efficient and preventative AD treatment.

Video Link

The video component of this article can be found at <https://www.jove.com/video/58229/>

Introduction

Amyloid- β (A β) deposits are found in the brain of patients with Alzheimer's disease (AD) and are considered a critical cause of AD¹ that disrupt neuronal networks, leading to memory impairments^{2,3,4}. Many clinical drug candidates have been shown to effectively prevent amyloid- β (A β) production or remove A β deposits. However, none have succeeded in improving memory function in AD patients⁵. A β is already deposited in the brain prior to the onset of memory impairments⁶; therefore, decreasing A β levels in the brains of patients exhibiting memory impairments may be ineffective. A β deposition is present in preclinical AD patients; however, these patients rarely present with neuronal degeneration and memory deficits⁶. There is a time lag between A β deposition and memory impairments. Therefore, a critical strategy for the prevention of AD is blocking A β toxicity signaling during the early stages of AD, prior to the development of memory deficits. A β deposition induces axon degeneration^{7,8,9,10,11,12,13}, which may lead to a disruption of neural networks and permanent impairment of memory function. Many studies have investigated the mechanisms of A β toxicity; for example, the degenerated axons of AD mice brains have been shown to have increased autophagy¹⁴. Calcineurin activation has been reported as a possible mechanism of A β -induced axonal degeneration¹⁵; however, the direct trigger of axonal degeneration remains unknown.

This study focuses on the collapse of axonal endings called growth cones. The collapse of axonal growth cones can be caused by axonal growth repellents, such as semaphorin-3A and ephrin-A5^{16,17,18,19,20}. Collapse-like dystrophic axonal endings have been observed in the brains of AD patients^{21,22}. Additionally, a failure of growth cone functioning can provoke axonal degeneration²³. However, it is unknown whether A β induces growth cone collapse. Therefore, this study presents a novel protocol to observe the early effects of A β in cultured neurons and investigate A β -induced growth cone collapse.

Protocol

All experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals at the Sugitani Campus of the University of Toyama and were approved by the Committee for Animal Care and Use of Laboratory Animals at the Sugitani Campus of the University of Toyama (A2014INM-1, A2017INM-1).

1. Collapse Assay

1. Poly-D-lysine coating
 1. Coat 8-well culture slides with 400 μ L of 5 μ g/mL poly-D-lysine (PDL) in phosphate-buffered saline (PBS) and incubate them at 37 °C overnight.

2. Remove the PDL solution and wash the wells 3 times with distilled water.
2. Neuron culture²⁴
 1. Mince freshly isolated cerebral cortices from embryonic day 14 (E14) ddY mice with microscissors in neuron culture medium containing 12% horse serum, 0.6% glucose, and 2 mM L-glutamine (medium A). Do not add antibiotics.
NOTE: In this protocol, the ddY mouse is used. This is an outbred strain commonly used in Japan. This neuron culture protocol can be also applied for rat cortical neurons^{7,25}.
 2. Centrifuge the tissues at 87 x g for 3 min.
 3. Remove the supernatant. Then to the pellet, add 2 mL of 0.05% trypsin and incubate for 15 min at 37°C. Mix by tapping every 5 min.
 4. Add 4 mL of medium A and mix by tapping.
 5. Centrifuge the tissues at 178 x g for 3 min.
 6. Remove the supernatant, and incubate the tissues with 600 U/mL DNase I and 0.3 mg/mL soybean trypsin inhibitor dissolved in PBS for 15 min at 37 °C. Mix by tapping every 5 min.
 7. After incubation, add 4 mL of medium A and mix by tapping.
 8. Centrifuge the tissues at 178 x g for 3 min.
 9. After removing the supernatant, add 4 mL of medium A and triturate the tissues with a polished Pasteur pipette.
 10. Filter the triturated tissues with a 70 µm pore-size mesh. After filtration, calculate the density of cells with a hemocytometer.
 11. Culture the cells in the 8-well culture slide at 0.8×10^4 cells/well with medium A and maintain them in a CO₂ incubator with a humidified atmosphere of 10% CO₂ at 37 °C.
 12. After 4 h of culturing, replace the culture medium to one containing 2% supplement for neuronal culture, 0.6% glucose, and 2 mM L-glutamine (medium B).
NOTE: The purity of neurons was approximately 75%, as described previously²⁶.
3. Collapse assay²⁷
 1. Dissolve commercially obtained full-length amyloid β 1-42 (A β 1-42) in distilled water at a concentration of 0.5 mM and incubate at 37 °C for 7 days. After the incubation, store the aggregated A β 1-42 solution in a -30 °C freezer until use.
NOTE: This incubation is necessary for aggregation and toxicity of A β ^{27,28,29,30}.
 2. After 4 days of neuronal culture, treat the wells with 100 µL of new medium B, containing 0.5 µM aggregated A β 1-42 or vehicle solution (distilled water) for 1 h.
NOTE: Effects of A β 1-42 were dose-dependently increased from 0.1 to 5 µM, and peaked at 0.5 µM as described previously²⁷. Similar results can be observed when by A β 1-42 treatment for 1 h after 3 days of neuronal culture³¹.
 3. Remove the culture medium and immediately fix the neurons with 4% paraformaldehyde containing 4% sucrose in PBS for 1 h at 37 °C on a hot plate.
 4. After fixation, wash the neurons 3 times with PBS and mount them with an aqueous mounting medium. Dry the mounting medium at 4 °C for 2–4 days.
 5. Capture the entire area (7.8 x 9 mm²) of each well with a 20X dry objective lens on an inverted microscope.
 6. Classify the longest neurites of each neuron in stage 3 or 4 as axons, as previously described^{32,33}.
 7. Classify growth cones according to the following criteria: 1) axonal growth cones lacking lamellipodia or 2) possessing fewer than three filopodia are considered collapsed growth cones, as described previously¹⁷.
NOTE: Healthy growth cones are scored as 0 point; collapsed growth cones are scored as 1 point. Mean collapse scores are calculated for each treatment.

2. Amyloid β Immunostaining

1. Culture mouse cortical neurons for 3 days, as described in step 1.2.
2. Treat with aggregated A β 1-42 (5 µM) or vehicle for 4 h at 37 °C in a CO₂ incubator.
3. Without removing the medium, add an equal volume of 4% paraformaldehyde containing 4% sucrose in PBS to each well, and maintain the culture at 37 °C on a hot plate for 5 min.
4. Replace the solution with 400 µL of 4% paraformaldehyde containing 4% sucrose in PBS, and maintain at 37 °C on the hot plate for 1 h. This fixation protocol was modified from a previous report³⁴.
5. Wash the neurons 3 times with PBS.
6. Block with 5% normal goat serum in PBS.
7. Incubate the neurons with mouse anti-amyloid β immunoglobulin G (IgG) (1:50) and 1% bovine serum albumin in PBS at 4 °C overnight.
8. Wash the neurons 3 times with PBS.
9. Incubate the neurons with a fluorescence-conjugated secondary antibody (1:400) and 1% bovine serum albumin in PBS at room temperature for 2 h.
10. Wash the neurons 3 times with PBS and mount them with an aqueous mounting medium.
11. Capture fluorescence images and bright field images with oblique illumination by using a 40X dry objective lens on inverted microscope B.

3. Axonal Immunostaining²⁷

1. Wash the neurons 3 times with PBS after cultured neuron fixation, as described in step 1.3.3.
2. Incubate the neurons with mouse anti-tau-1 IgG (1:500), rabbit anti-microtubule associated protein 2 (MAP2) IgG (1:500) in 5% normal goat serum, and 0.3% *t*-octylphenoxypoly-ethoxyethanol in PBS at 4 °C overnight.
3. Wash the neurons 3 times with PBS.
4. Incubate the neurons with fluorescence-conjugated secondary antibodies (1:400) and 0.3% *t*-octylphenoxypolyethoxyethanol in PBS at room temperature for 2 h.
5. Wash the neurons 3 times with PBS, and mount using an aqueous mounting medium.

- Capture fluorescence and differential interference contrast (DIC) images by using a 20× dry objective lens on inverted microscope A.

4. Live Cell Imaging²⁷

- Coat glass-based dishes with 500 μL of PDL (5 $\mu\text{g}/\text{mL}$), as described in step 1.1.1.
NOTE: In this protocol, homemade glass-based dishes were used. Commercially available glass-based dishes can also be used for live imaging. Homemade glass-based dishes were prepared as follows: 1) make a hole approximately 1.4 mm in diameter in the center of a 35-mm dish with a hand punch, and 2) attach a glass coverslip (diameter of 22 mm) to the back of the dish with silicone.
- Wash the plates with distilled water, as described in step 1.1.2, and culture the cortical neurons in the glass-based dish at 3×10^4 cells/dish with medium A, as described in step 1.2.
- After 4 days of cell culture, replace the medium with 2 mL of new medium B, and transfer the dish to inverted microscope A. Maintain the culture in a humidified atmosphere of 10% CO_2 at 37 °C.

5. Endocytosis Experiment

- Culture the mouse cortical neurons as described in step 1.2.
- Four days later, replace the medium with 100 μL of new medium B containing 20 μM fluorescence membrane probe for 1 min.
- Add 1 μL of 0.05 mM aggregated A β 1-42 (final 0.5 μM) or vehicle (distilled water) solution and mix by pipetting. Incubate for 20 min.
- Remove the medium and wash the wells twice with medium B that has been pre-warmed to 37 °C.
- Fix, wash, and mount the neurons as described in steps 1.3.3 and 1.3.4.
- Capture fluorescent and DIC images with a 63X oil objective lens on inverted microscope A.
- Quantify the density of the fluorescence membrane probe-positive area in each healthy growth cone by using an image software.

6. Gene Transfection

- Prepare cortical neurons as described in step 1.2. After completing steps 1.2.1 to 1.2.10, centrifuge the neurons at 178 x g for 3 min.
- Remove the supernatant, add 4 mL of Ca^{2+} -free and Mg^{2+} -free Hanks' balanced salt solution (CMF-HBSS), and mix by pipetting.
- Centrifuge the cells at 178 x g for 3 min.
- Remove the supernatant, add 4 mL of CMF-HBSS, and mix by pipetting. Next, calculate the cell density, as described in step 1.2.10.
- Transfer 5×10^6 cells to a 1.5 mL tube and centrifuge at 1,677 x g for 1 min.
- Remove the supernatant, add 100 μL of transfection solution with supplement and 3 μg of DNA plasmid encoding EGFP or EGFP-AP180 C-terminus, and mix by pipetting.
- Transfer the above solution (step 6.6) to a certified cuvette and transfect with an electroporator, according to the manufacturer's protocol.
- Immediately after transfection, add 500 μL of medium A into the cuvette and transfer the solution to a 1.5-mL tube with a certified pipette. Next, calculate the cell density, as described in step 1.2.10.
- Culture the cells in an 8-well culture slide at 0.8×10^4 cells/well, as described in steps 1.2.11 and 1.2.12.
- After 4 days of cell culture, perform a collapse assay as described in step 1.3.

Representative Results

In this protocol, A β 1-42 was incubated at 37 °C for 7 days before use, because incubation of A β 1-42 was needed for producing toxic forms^{27,28,30,35}. After this incubation, aggregated forms of A β were observed (**Figure 1A**). It has been reported that similar incubation of A β 1-42 produced the fibril form of A β ³⁶. After treatment with this aggregated A β 1-42, immunostaining with an antibody for the toxic oligomer of A β ^{35,37} was performed, and positive staining was detected on cultured neurons (**Figure 1B**). Considering the above, this incubation protocol produces the toxic forms of A β .

Several days were required for the induction of axonal degeneration after A β exposure. The events prior to axonal degeneration remain unclear. Therefore, this protocol has been developed to further understand the mechanisms involved. Using this protocol, the early phenomena induced by A β treatment were analyzed. Cortical neurons were cultured for 4 days. The longest neurites in the cultured neurons were identified as axons; these were confirmed by positive immunostaining for the axonal marker, tau-1, and negative immunostaining for the dendritic marker, MAP2 (**Figure 2**). After 1 h of vehicle treatment, growth cones had spread lamellipodia and processed several filopodia. These were identified as healthy growth cones. Conversely, 1 h of A β 1-42 treatment led to shrunken growth cones, which developed no lamellipodia or filopodia. These were identified as collapsed growth cones. Collapse scores were calculated as described in step 1.3.7. When shapes of growth cones were unclear, they were eliminated from the analysis. A β 1-42 treatment led to a significant increase in collapse score, corresponding to increased axonal growth collapse, when compared to the collapse score of vehicle-treated growth cones²⁷.

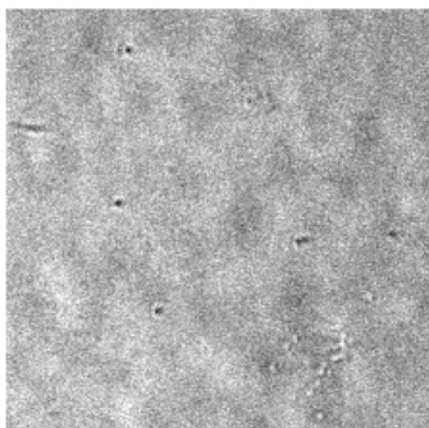
Axonal growth cones were observed before and after treatment with A β 1-42 (**Figure 3**). Cells were maintained in the inverted microscope with a humidified atmosphere of 10% CO_2 at 37 °C. Images were captured every 5 min. As shown in **Figure 3**, growth cones collapsed between 21 and 26 min after A β 1-42 treatment. Growth cones were excluded from live cell imaging if they did not retain their healthy shape for 1 h prior to any treatment.

To visualize the early effects of A β 1-42-treatment, endocytosis was used as the focus of this analysis, because endocytosis inhibitors can block A β 1-42-induced growth-cone collapse²⁷. Endocytosis was visualized with a fluorescence membrane probe (*i.e.*, a fluorescent dye that binds to plasma membranes and is spontaneously endocytosed). A previous study showed that growth cones do not collapse at 20 min after A β 1-42-treatment²⁷; therefore, healthy growth cones were selected by DIC imaging in vehicle- or A β 1-42-treated cells after 20 min. Following A β 1-42-treatment, numerous fluorescent membrane probe-positive puncta were observed in the growth cone (**Figure 4**). The density of fluorescence membrane probe-positive puncta in growth cones was significantly increased²⁷. This suggests that A β 1-42-induced growth cone endocytosis occurs prior to collapse.

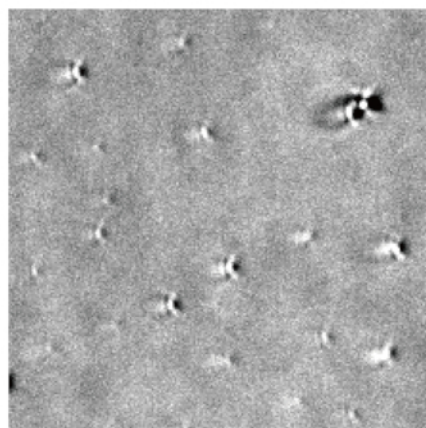
To confirm the role of endocytosis, a DNA plasmid encoding EGFP-AP180 C-terminus was transfected into cultured cortical neurons. Cells expressing the AP180 C-terminus selectively inhibited clathrin-mediated endocytosis^{38,39}. If EGFP expression was observed at the cell body in the neuron, the AP180 C-terminus was considered to be expressed at the axonal growth cone of the neuron. Transfection of AP180 C-terminus blocked A β 1-42-induced growth cone collapse (**Figure 5**)²⁷.

A

No incubation

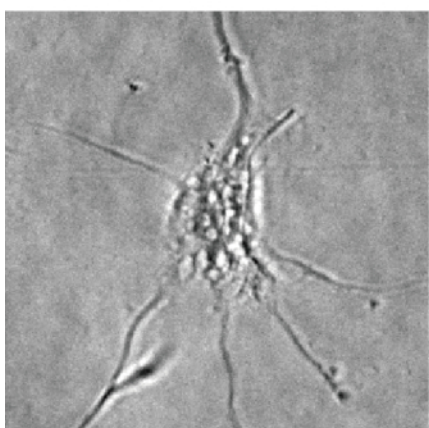


After incubation



B

Vehicle



Aβ1-42

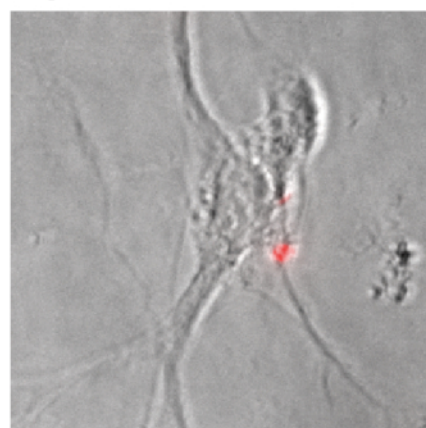


Figure 1: Incubation of Aβ1-42 aggregates Aβ. (A) Aβ1-42 was dissolved in distilled water at a concentration of 0.5 mM and incubated at 37 °C for 7 days (after incubation), or stored at -30 °C without incubation (no incubation). Each Aβ solution was diluted to 0.1 mM; then, 10 μL of each diluted solution was dropped on glass slides and covered with coverslips. Bright-field images with oblique illumination were captured by using inverted microscope B. Scale bar = 20 μm. (B) Aggregated Aβ1-42 or vehicle treatment on cultured neurons for 4 h. Following treatment, the neurons were fixed and immunostained for toxic Aβ oligomers. Fluorescence images (red) and bright-field images with oblique illumination (gray) are shown. Scale bar = 20 μm. [Please click here to view a larger version of this figure.](#)

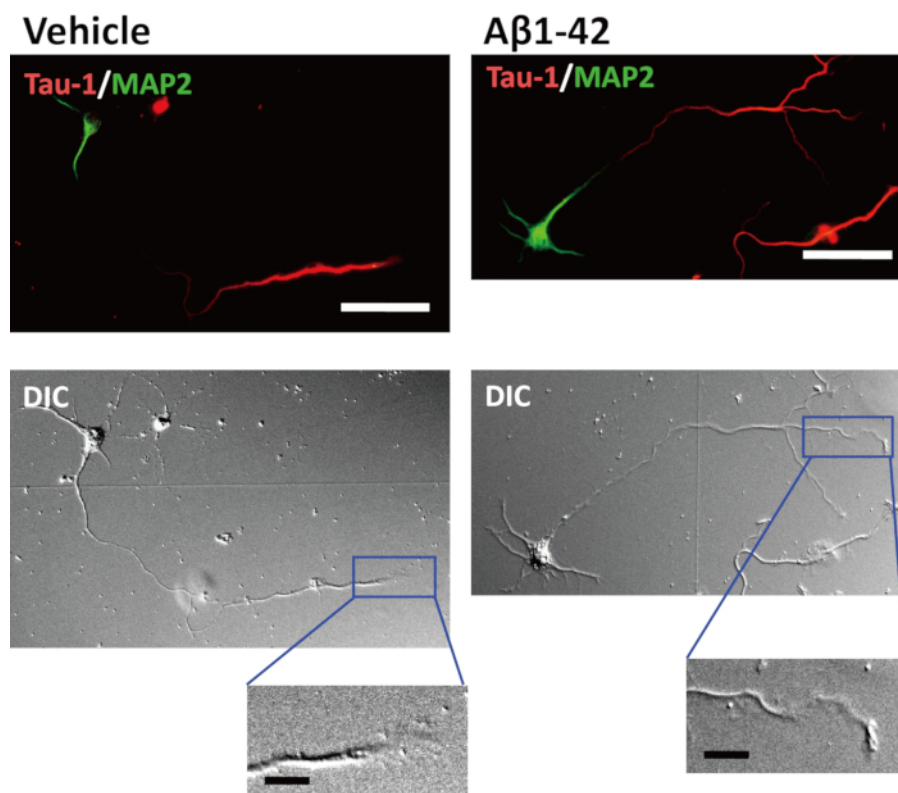


Figure 2: A β 1-42-induced axonal growth cone collapse. After A β 1-42- or vehicle-treatment, neurons were fixed and immunostained for tau-1 (red) and microtubule associated protein 2 (MAP2, green). Fluorescence and differential interference contrast (DIC) images are shown. Magnified views of the regions of interest (ROI, rectangles) are shown below their corresponding images. White scale bars = 50 μ m; black scale bars = 10 μ m. This figure has been modified from Kuboyama et al, 2015²⁷. [Please click here to view a larger version of this figure.](#)

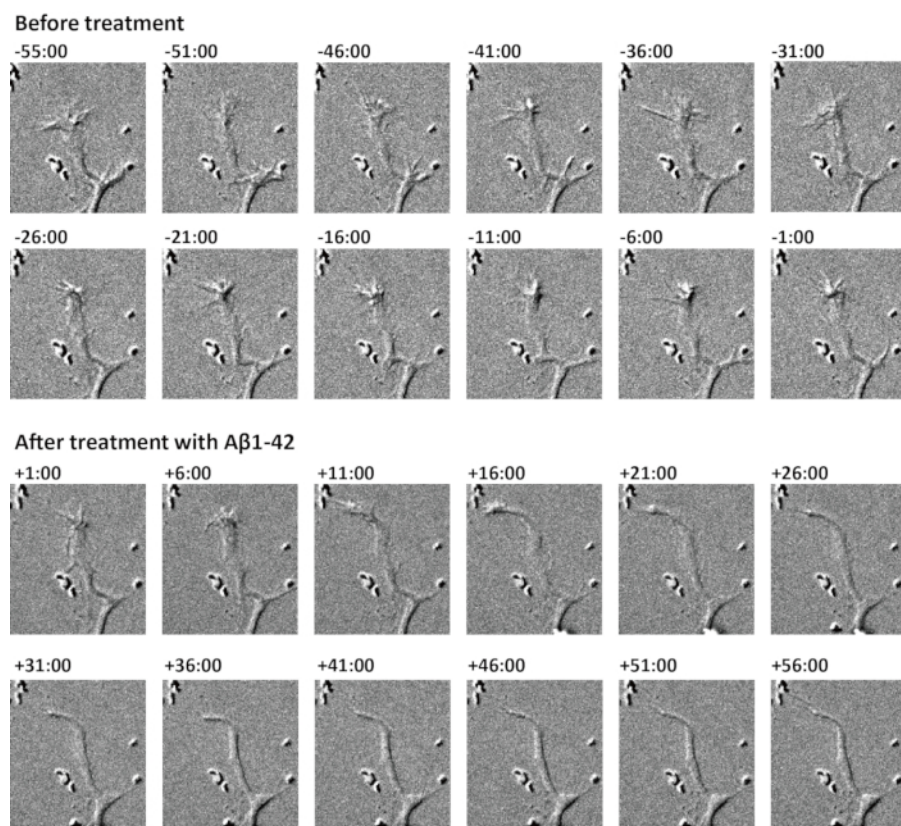
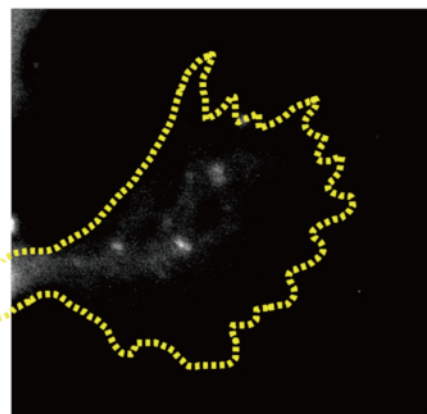


Figure 3: Live cell imaging before and after A β 1-42 treatment. After 4 days of culture, cells were transferred to an inverted microscope and DIC images were captured every 5 min. Time-lapse images are shown. The digits represent minutes:seconds after the application of aggregated A β 1-42 (final concentration, 0.5 μ M). Scale bar = 10 μ m. [Please click here to view a larger version of this figure.](#)

Vehicle



A β 1-42

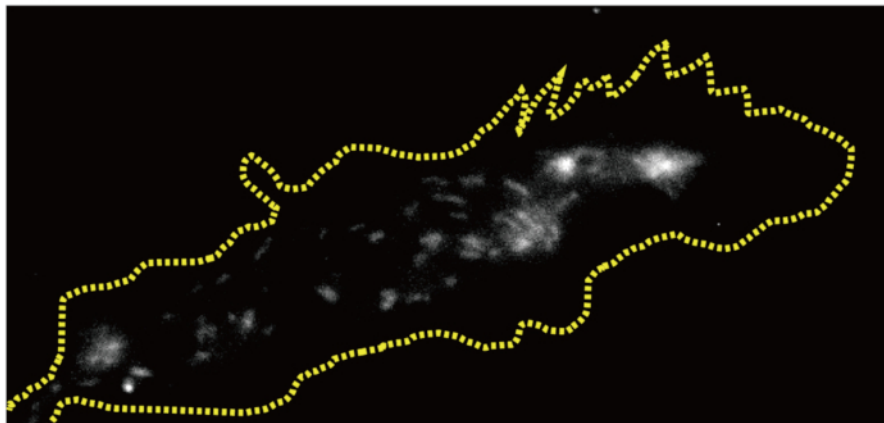


Figure 4: Twenty minutes of A β 1-42 treatment induced endocytosis. Cortical neurons were cultured for 4 days and treated with a fluorescence membrane probe. Then, neurons were treated for 20 min with A β 1-42 or vehicle. Fluorescence images of the growth cones are shown. The yellow dotted lines represent the outlines of the growth cones. Scale bar = 10 μ m. [Please click here to view a larger version of this figure.](#)

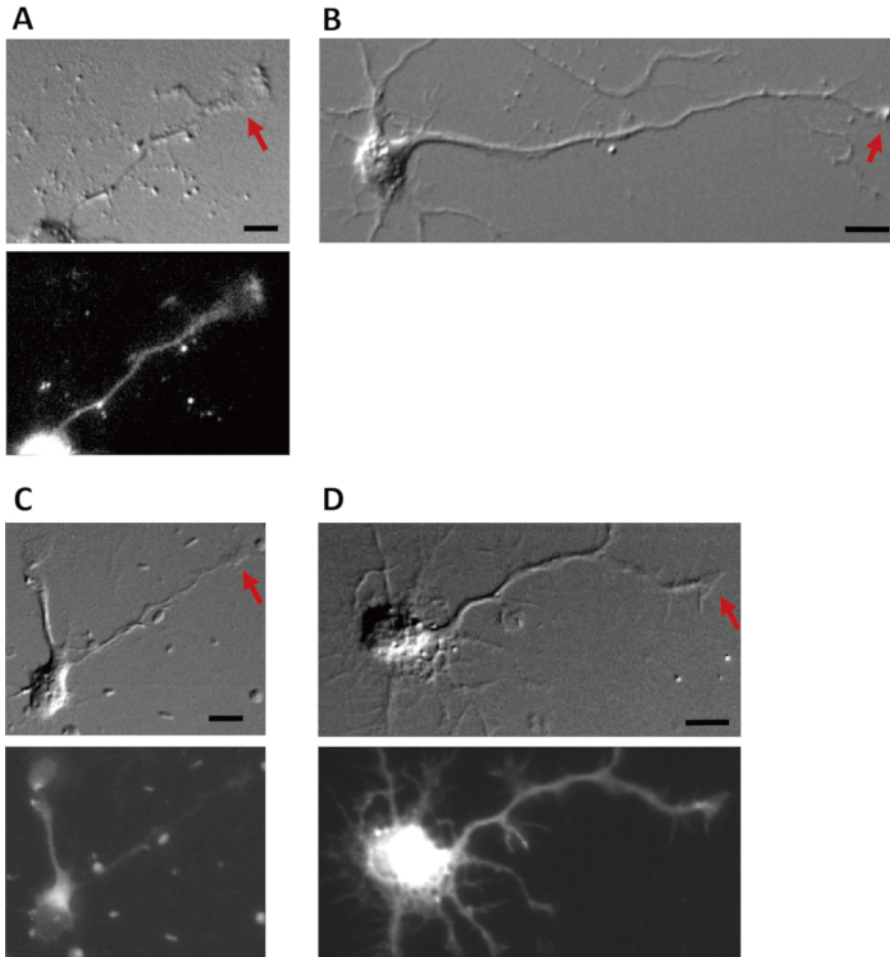


Figure 5: Expression of AP180-C terminus blocks Aβ1-42-induced collapse. Four days after transfection of EGFP (A, B) or EGFP-AP180 C-terminus (C, D); Aβ1-42 (B, D) or vehicle (A, C) was added to cortical neurons for 1 h. DIC (upper panels) and fluorescence (bottom panels) images are shown. Arrows indicate growth cones. Scale bars = 10 μm. [Please click here to view a larger version of this figure.](#)

Discussion

The protocol described in this study enabled the observation of early phenomena in axonal growth cones after Aβ1-42 treatment. Aβ1-42 induced endocytosis in axonal growth cones within 20 min, and growth cone collapse was observed within 1 h of treatment. This endocytosis was probably mediated by clathrin. By using this protocol, the inhibition of clathrin-mediated endocytosis was confirmed to prevent Aβ1-42-induced growth cone collapse and axonal degeneration in cultured neurons²⁷. Additionally, the inhibition of clathrin-mediated endocytosis attenuated Aβ1-42-induced axonal degeneration and memory deficits *in vivo*²⁷. These results indicate that clathrin-mediated endocytosis is a promising therapeutic avenue for AD prevention.

This protocol was developed from collapse assays for axonal growth repellents, such as semaphorin 3A and ephrin-A5^{16,17,18,19,20}. Collapse assays have been used in studies assessing the development of neuronal networks. I have shown that this protocol can be applied to pathological analyses, particularly those involving mechanisms of AD; however, a limitation may be that approximately 40% of growth cones collapsed in the healthy condition. This percentage is higher than results from cultured dorsal root ganglion neurons, which are more commonly used in collapse assays^{16,17,18,19,20}. Therefore, the difference in cell types might be linked to differences in collapse ratios. The collapse ratios found in this study were consistent with those found in previous studies with normal cultured cortical neurons^{40,41}. Furthermore, Aβ1-42 induced similar levels of growth cone collapse when compared with other collapse factors, such as semaphorin 3A and ephrin-A5²⁷. Therefore, this protocol is valid for the quantification of Aβ1-42-induced growth cone collapse. This fixation protocol is important to maintain the shape of growth cones. If the cells were conventionally fixed with 4% paraformaldehyde at room temperature, more growth cones may have collapsed due to the fixation procedure (data not shown). Alternatively, glutaraldehyde and fixation buffers are available for rigid fixation, as previously described⁴²; however, glutaraldehyde exhibits autofluorescence, which is a significant impediment for fluorescence imaging.

A recent study with the same protocol showed that the water extract from *Radix Polygalae* (roots of *Polygala tenuifolia*) inhibited Aβ1-42-induced endocytosis in cultured neurons, prevented axonal degeneration, and reduced memory deficits in a transgenic mouse model of AD³¹. A novel candidate for AD prevention has been found with this protocol. A combination of gene transfection and live cell imaging in this protocol might show the other cellular events found in axons and their terminals before and after Aβ treatment, such as Ca²⁺ imaging, microtubule dynamics, and cell adhesion dynamics, which are reportedly related to axonal growth^{43,44,45}. This protocol may help reveal more detailed mechanisms of Aβ toxicity and may help lead to the prevention and/or treatment of AD.

Disclosures

The author has nothing to disclose.

Acknowledgements

This work was partially supported by research grants from JSPS (KAKENHI 18K07389), Japan, Takeda Science Foundation, Japan, and Kobayashi Pharmaceutical Co., Ltd., Japan.

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