**TITLE:**

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

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**SUMMARY:**

Here a protocol to investigate the early effects of amyloid-β (Aβ) in the brain is presented. This shows that Aβ induces clathrin-mediated endocytosis and collapse of axonal growth cones. The protocol is useful in studying early effects of Aβ on axonal growth cones and may facilitate prevention of Alzheimer’s disease.

**ABSTRACT:**

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

**INTRODUCTION:**

Amyloid-β (Aβ) 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 impairments2-4. Many clinical drug candidates have been shown to effectively prevent amyloid-β (Aβ) production or remove Aβ deposits. However, none have succeeded in improving memory function in AD patients5. Aβ is already deposited in the brain prior to the onset of memory impairments6; therefore, decreasing Aβ levels in the brains of patients exhibiting memory impairments may be ineffective. Aβ deposition is present in preclinical AD patients; however, these patients rarely present with neuronal degeneration and memory deficits6. There is a time lag between Aβ deposition and memory impairments. Therefore, a critical strategy for the prevention of AD is blocking Aβ toxicity signaling during the early stages of AD, prior to the development of memory deficits. Aβ deposition induces axon degeneration7-13, which may lead to a disruption of neural networks and permanent impairment of memory function. Many studies have investigated the mechanisms of Aβ 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 Aβ-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-A516-20. Collapse-like dystrophic axonal endings have been observed in the brains of AD patients21,22. Additionally, a failure of growth cone functioning can provoke axonal degeneration23. However, it is unknown whether Aβ induces growth cone collapse. Therefore, this study presents a novel protocol to observe the early effects of Aβ in cultured neurons and investigate Aβ-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**

1.1. Poly-D-lysine coating

1.1.1. Coat 8-well culture slides with 400 μL of 5 μg/mL poly-D-lysine (PDL) in phosphate-buffered saline (PBS) and incubate them at 37 °C overnight.

1.1.2. Remove the PDL solution and wash the wells 3 times with distilled water.

1.2. Neuron culture24

1.2.1. 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 neurons7,25.

1.2.2. Centrifuge the tissues at 87 x g for 3 min.

1.2.3. 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.

1.2.4. Add 4 mL of medium A and mix by tapping.

1.2.5. Centrifuge the tissues at 178 x g for 3 min.

1.2.6. 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.

1.2.7. After incubation, add 4 mL of medium A and mix by tapping.

1.2.8. Centrifuge the tissues at 178 x g for 3 min.

1.2.9. After removing the supernatant, add 4 mL of medium A and triturate the tissues with a polished Pasteur pipette.

1.2.10. Filter the triturated tissues with a 70-µm pore-size mesh. After filtration, calculate the density of cells with a hemocytometer.

1.2.11. Culture the cells in the 8-well culture slide at 0.8 x 104 cells/well with medium A and maintain them in a CO2 incubator with a humidified atmosphere of 10% CO2 at 37 °C.

1.2.12. 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.

1.3. Collapse assay27

1.3.1. Dissolve commercially obtained full-length amyloid β1-42 (Aβ1-42) in distilled water at a concentration of 0.5 mM and incubate at 37 °C for 7 days. After the incubation, store the aggregated Aβ1-42 solution in a -30 °C freezer until use.

Note: This incubation is necessary for aggregation and toxicity of Aβ27-30.

1.3.2. After 4 days of neuronal culture, treat the wells with 100 μL of new medium B, containing 0.5 μM aggregated Aβ1-42 or vehicle solution (distilled water) for 1 h.

Note: Effects of Aβ1-42 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 Aβ1-42 treatment for 1 h after 3 days of neuronal culture31.

1.3.3. 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.

1.3.4. 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.

1.3.5. Capture the entire area (7.8 x 9 mm) of each well with a 20X dry objective lens on an inverted microscope.

1.3.6. Classify the longest neurites of each neuron in stage 3 or 4 as axons, as previously described32,33.

1.3.7. 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**

2.1. Culture mouse cortical neurons for 3 days, as described in step 1.2.

2.2. Treat with aggregated Aβ1-42 (5 μM) or vehicle for 4 h at 37 °C in a CO2 incubator.

2.3. 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.

2.4. 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.

2.5. Wash the neurons 3 times with PBS.

2.6. Block with 5% normal goat serum in PBS.

2.7. Incubate the neurons with mouse anti-amyloid β immunoglobulin G (IgG) (1:50) and 1% bovine serum albumin in PBS at 4 °C overnight.

2.8. Wash the neurons 3 times with PBS.

2.9. Incubate the neurons with a fluorescence-conjugated secondary antibody (1:400) and 1% bovine serum albumin in PBS at room temperature for 2 h.

2.10. Wash the neurons 3 times with PBS and mount them with an aqueous mounting medium.

2.11. Capture fluorescence images and bright field images with oblique illumination by using a 40X dry objective lens on inverted microscope B.

**3. Axonal Immunostaining27**

3.1. Wash the neurons 3 times with PBS after cultured neuron fixation, as described in step 1.3.3.

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.

3.3. Wash the neurons 3 times with PBS.

3.4. Incubate the neurons with fluorescence-conjugated secondary antibodies (1:400) and 0.3% *t*-octylphenoxypolyethoxyethanol in PBS at room temperature for 2 h.

3.5. Wash the neurons 3 times with PBS, and mount using an aqueous mounting medium.

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

**4. Live Cell Imaging27**

4.1 Coat glass-based dishes with 500 μL of PDL (5 μg/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.

4.2. 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 x 104 cells/dish with medium A, as described in step 1.2.

4.3. 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% CO2 at 37 °C.

**5. Endocytosis Experiment**

5.1. Culture the mouse cortical neurons as described in step 1.2.

5.2. Four days later, replace the medium with 100 μL of new medium B containing 20 μM fluorescence membrane probe for 1 min.

5.3. Add 1 μL of 0.05 mM aggregated Aβ1-42 (final 0.5 μM) or vehicle (distilled water) solution and mix by pipetting. Incubate for 20 min.

5.4. Remove the medium and wash the wells twice with medium B that has been pre-warmed to 37 °C.

5.5. Fix, wash, and mount the neurons as described in steps 1.3.3 and 1.3.4.

5.6. Capture fluorescent and DIC images with a 63X oil objective lens on inverted microscope A.

5.7. Quantify the density of the fluorescence membrane probe-positive area in each healthy growth cone by using an image software.

**6. Gene Transfection**

6.1. 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.

6.2. Remove the supernatant, add 4 mL of Ca2+-free and Mg2+-free Hanks' balanced salt solution (CMF-HBSS), and mix by pipetting.

6.3. Centrifuge the cells at 178 x g for 3 min.

6.4. 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.

6.5. Transfer 5 x 106 cells to a 1.5 mL tube and centrifuge at 1,677 x g for 1 min.

6.6. 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.

6.7. Transfer the above solution (step 6.6) to a certified cuvette and transfect with an electroporator, according to the manufacturer’s protocol.

6.8. 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.

6.9. Culture the cells in an 8-well culture slide at 0.8 x 104 cells/well, as described in steps 1.2.11 and 1.2.12.

6.10. After 4 days of cell culture, perform a collapse assay as described in step 1.3.

**REPRESENTATIVE RESULTS:**

In this protocol, Aβ1-42 was incubated at 37 °C for 7 days before use, because incubation of Aβ1-42 was needed for producing toxic forms27,28,30,35. After this incubation, aggregated forms of Aβ were observed (**Figure 1A**). It has been reported that similar incubation of Aβ1-42 produced the fibril form of Aβ36. After treatment with this aggregated Aβ1-42, immunostaining with an antibody for the toxic oligomer of Aβ35,37 was performed, and positive staining was detected on cultured neurons (**Figure 1B**). Considering the above, this incubation protocol produces the toxic forms of Aβ.

Several days were required for the induction of axonal degeneration after Aβ 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 Aβ 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 Aβ1-42 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. Aβ1-42 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 Aβ1-42 (**Figure 3**). Cells were maintained in the inverted microscope with a humidified atmosphere of 10% CO2 at 37 °C. Images were captured every 5 min. As shown in **Figure 3**, growth cones collapsed between 21 and 26 min after Aβ1-42 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 Aβ1-42-treatment, endocytosis was used as the focus of this analysis, because endocytosis inhibitors can block Aβ1-42-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 Aβ1-42-treatment27; therefore, healthy growth cones were selected by DIC imaging in vehicle- or Aβ1-42-treated cells after 20 min. Following Aβ1-42-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 Aβ1-42-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 endocytosis38,39. 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 Aβ1-42-induced growth cone collapse (**Figure 5**)27.

**FIGURE AND TABLE LEGENDS:**

**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.

**Figure 2**: **Aβ1-42-induced axonal growth cone collapse.** After Aβ1-42- 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 μm; black scale bars = 10 μm. This figure has been modified from Kuboyama et al, 201527.

**Figure 3:** **Live cell imaging before and after Aβ1-42 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 Aβ1-42 (final concentration, 0.5 μM). Scale bar = 10 μm.

**Figure 4:** **Twenty minutes of Aβ1-42 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 Aβ1-42 or vehicle. Fluorescence images of the growth cones are shown. The yellow dotted lines represent the outlines of the growth cones. Scale bar = 10 μm.

**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.

**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-A516-20. 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 assays16-20. 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 neurons40,41. 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 growth43-45. This protocol may help reveal more detailed mechanisms of Aβ toxicity and may help lead to the prevention and/or treatment of AD.

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**DISCLOSURES:**

The author has nothing to disclose.

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