Journal of Visualized Experiments

Visualizing Early Effects of Amyloid β, Such as Axonal Growth Cone Collapse, in Mouse Cultured Neurons.

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58229R2
Full Title:	Visualizing Early Effects of Amyloid β, Such as Axonal Growth Cone Collapse, in Mouse Cultured Neurons.
Keywords:	amyloid β; axon; growth cone; collapse; endocytosis; live cell imaging
Corresponding Author:	T Dr. Kuboyama
Corresponding Author's Institution:	
Corresponding Author E-Mail:	kuboyama@inm.u-toyama.ac.jp
Order of Authors:	Tomoharu Kuboyama
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	University of Toyama, Sugitani 2630, Toyama 9300194, Japan

Powered by Editorial Manager® and ProduXion Manager® from Aries Systems Corporation

Manuscript Click here to download Manuscript 58229_R2_RE.docx

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

AUTHORS & AFFILIATIONS:
Tomoharu Kuboyama[1]
[1]Division of Neuromedical Science, Institute of Natural Medicine, University of Toyama, Toyama, Japan

Corresponding Author:
Tomoharu Kuboyama
Tel: +81-76-434-7670
kuboyama@inm.u-toyama.ac.jp

KEYWORDS:
Amyloid β, axon, growth cone, collapse, endocytosis, live cell imaging

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 AD[1] that disrupt neuronal networks, leading to memory impairments[2-4]. 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 patients[5]. Aβ is already deposited in the brain prior to the onset of memory

impairments[6]; 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 deficits[6]. 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 degeneration[7-13], 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 autophagy[14]. Calcineurin activation has been reported as a possible mechanism of Aβ-induced axonal degeneration[15]; 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[16-20]. 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 degeneration[23]. 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 culture[24]

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 neurons[7,25].

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 0.8×10^4 cells/well with medium A and maintain them in a CO_2 incubator with a humidified atmosphere of 10% CO_2 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 previously[26].

1.3. Collapse assay[27]

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 Aβ[27-30].

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 previously[27]. Similar results can be observed when by Aβ1-42 treatment for 1 h after 3 days of neuronal culture[31].

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 described[32,33].

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 previously[17].

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 CO_2 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 report[34].

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 Immunostaining[27]

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 Imaging[27]

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 10^4 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% CO_2 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 Ca^{2+}-free and Mg^{2+}-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 5×10^6 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 0.8×10^4 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 forms[27,28,30,35]. 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 Aβ[36]. After treatment with this aggregated Aβ1-42, immunostaining with an antibody for the toxic oligomer of Aβ[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 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 cones[27].

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% 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 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 collapse[27]. 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-treatment[27]; 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 increased[27]. 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 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 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, 2015[27].

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 neurons[27]. 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[16-20]. 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[16-20]. 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-A5[27]. 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 described[42]; 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 AD[31]. 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 Ca^{2+} imaging, microtubule dynamics, and cell adhesion dynamics, which are reportedly related to axonal growth[43-45]. This protocol may help reveal more detailed mechanisms of Aβ toxicity and may help lead to the prevention and/or treatment of AD.

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

DISCLOSURES:
The author has nothing to disclose.

REFERENCES:
1 Selkoe, D. J. & Hardy, J. The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO Molecular Medicine.* **8** (6), 595-608, doi:10.15252/emmm.201606210, (2016).
2 Dickson, T. C. & Vickers, J. C. The morphological phenotype of beta-amyloid plaques and

associated neuritic changes in Alzheimer's disease. *Neuroscience.* **105** (1), 99-107 (2001).

3 Hardy, J. & Selkoe, D. J. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science.* **297** (5580), 353-356, doi:10.1126/science.1072994, (2002).

4 Perl, D. P. Neuropathology of Alzheimer's disease. *Mount Sinai Journal of Medicine.* **77** (1), 32-42, doi:10.1002/msj.20157, (2010).

5 Graham, W. V., Bonito-Oliva, A. & Sakmar, T. P. Update on Alzheimer's Disease Therapy and Prevention Strategies. *Annual Review of Medicine.* **68** 413-430, doi:10.1146/annurev-med-042915-103753, (2017).

6 Jack, C. R., Jr. *et al.* Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade. *Lancet Neurology.* **9** (1), 119-128, doi:10.1016/s1474-4422(09)70299-6, (2010).

7 Kuboyama, T., Tohda, C. & Komatsu, K. Neuritic regeneration and synaptic reconstruction induced by withanolide A. *British Journal of Pharmacology.* **144** (7), 961-971, doi:10.1038/sj.bjp.0706122, (2005).

8 Tohda, C., Urano, T., Umezaki, M., Nemere, I. & Kuboyama, T. Diosgenin is an exogenous activator of 1,25D_3-MARRS/Pdia3/ERp57 and improves Alzheimer's disease pathologies in 5XFAD mice. *Scientific Reports.* **2** 535, doi:10.1038/srep00535, (2012).

9 Jawhar, S., Trawicka, A., Jenneckens, C., Bayer, T. A. & Wirths, O. Motor deficits, neuron loss, and reduced anxiety coinciding with axonal degeneration and intraneuronal Abeta aggregation in the 5XFAD mouse model of Alzheimer's disease. *Neurobiology of Aging.* **33** (1), 196.e129-140, doi:10.1016/j.neurobiolaging.2010.05.027, (2012).

10 Postuma, R. B. *et al.* Substrate-bound beta-amyloid peptides inhibit cell adhesion and neurite outgrowth in primary neuronal cultures. *Journal of Neurochemistry.* **74** (3), 1122-1130 (2000).

11 Tohda, C., Nakada, R., Urano, T., Okonogi, A. & Kuboyama, T. Kamikihi-to (KKT) rescues axonal and synaptic degeneration associated with memory impairment in a mouse model of Alzheimer's disease, 5XFAD. *International Journal of Neuroscience.* **121** (12), 641-648, doi:10.3109/00207454.2011.602809, (2011).

12 Tsai, J., Grutzendler, J., Duff, K. & Gan, W. B. Fibrillar amyloid deposition leads to local synaptic abnormalities and breakage of neuronal branches. *Nature Neuroscience.* **7** (11), 1181-1183, doi:10.1038/nn1335, (2004).

13 Wirths, O., Weis, J., Kayed, R., Saido, T. C. & Bayer, T. A. Age-dependent axonal degeneration in an Alzheimer mouse model. *Neurobiology of Aging.* **28** (11), 1689-1699, doi:10.1016/j.neurobiolaging.2006.07.021, (2007).

14 Sanchez-Varo, R. *et al.* Abnormal accumulation of autophagic vesicles correlates with axonal and synaptic pathology in young Alzheimer's mice hippocampus. *Acta Neuropathologica.* **123** (1), 53-70, doi:10.1007/s00401-011-0896-x, (2012).

15 Wu, H. Y. *et al.* Amyloid beta induces the morphological neurodegenerative triad of spine loss, dendritic simplification, and neuritic dystrophies through calcineurin activation. *Journal of Neuroscience.* **30** (7), 2636-2649, doi:10.1523/jneurosci.4456-09.2010, (2010).

16 Campbell, D. S. & Holt, C. E. Chemotropic responses of retinal growth cones mediated by rapid local protein synthesis and degradation. *Neuron.* **32** (6), 1013-1026 (2001).

17 Jurney, W. M., Gallo, G., Letourneau, P. C. & McLoon, S. C. Rac1-mediated endocytosis

during ephrin-A2- and semaphorin 3A-induced growth cone collapse. *Journal of Neuroscience.* **22** (14), 6019-6028, doi:20026594, (2002).
18 Luo, Y., Raible, D. & Raper, J. A. Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. *Cell.* **75** (2), 217-227 (1993).
19 Nicol, X., Muzerelle, A., Rio, J. P., Metin, C. & Gaspar, P. Requirement of adenylate cyclase 1 for the ephrin-A5-dependent retraction of exuberant retinal axons. *Journal of Neuroscience.* **26** (3), 862-872, doi:10.1523/JNEUROSCI.3385-05.2006, (2006).
20 Wu, K. Y. *et al.* Local translation of RhoA regulates growth cone collapse. *Nature.* **436** (7053), 1020-1024, doi:10.1038/nature03885, (2005).
21 Benes, F. M., Farol, P. A., Majocha, R. E., Marotta, C. A. & Bird, E. D. Evidence for axonal loss in regions occupied by senile plaques in Alzheimer cortex. *Neuroscience.* **42** (3), 651-660 (1991).
22 Masliah, E. *et al.* An antibody against phosphorylated neurofilaments identifies a subset of damaged association axons in Alzheimer's disease. *American Journal of Pathology.* **142** (3), 871-882 (1993).
23 Zhou, F. Q. & Snider, W. D. Intracellular control of developmental and regenerative axon growth. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences.* **361** (1473), 1575-1592, doi:10.1098/rstb.2006.1882, (2006).
24 Teshigawara, K. *et al.* A novel compound, denosomin, ameliorates spinal cord injury via axonal growth associated with astrocyte-secreted vimentin. *British Journal of Pharmacology.* **168** (4), 903-919, doi:10.1111/j.1476-5381.2012.02211.x, (2013).
25 Kuboyama, T., Tohda, C. & Komatsu, K. Withanoside IV and its active metabolite, sominone, attenuate A beta(25-35)-induced neurodegeneration. *European Journal of Neuroscience.* **23** (6), 1417-1426, doi:10.1111/j.1460-9568.2006.04664.x, (2006).
26 Tanabe, N., Kuboyama, T. & Tohda, C. Matrine Directly Activates Extracellular Heat Shock Protein 90, Resulting in Axonal Growth and Functional Recovery in Spinal Cord Injured-Mice. *Frontiers in Pharmacology.* **9** (446), doi:10.3389/fphar.2018.00446, (2018).
27 Kuboyama, T., Lee, Y. A., Nishiko, H. & Tohda, C. Inhibition of clathrin-mediated endocytosis prevents amyloid beta-induced axonal damage. *Neurobiology of Aging.* **36** (5), 1808-1819, doi:10.1016/j.neurobiolaging.2015.02.005, (2015).
28 Pike, C. J., Walencewicz, A. J., Glabe, C. G. & Cotman, C. W. In vitro aging of beta-amyloid protein causes peptide aggregation and neurotoxicity. *Brain Research.* **563** (1-2), 311-314 (1991).
29 Uchida, N. *et al.* Yokukansan inhibits social isolation-induced aggression and methamphetamine-induced hyperlocomotion in rodents. *Biological and Pharmaceutical Bulletin.* **32** (3), 372-375 (2009).
30 Pike, C. J., Burdick, D., Walencewicz, A. J., Glabe, C. G. & Cotman, C. W. Neurodegeneration induced by beta-amyloid peptides *in vitro*: the role of peptide assembly state. *Journal of Neuroscience.* **13** (4), 1676-1687 (1993).
31 Kuboyama, T., Hirotsu, K., Arai, T., Yamasaki, H. & Tohda, C. Polygalae Radix Extract Prevents Axonal Degeneration and Memory Deficits in a Transgenic Mouse Model of Alzheimer's Disease. *Frontiers in Pharmacology.* **8** 805, doi:10.3389/fphar.2017.00805, (2017).
32 Dotti, C. G., Sullivan, C. A. & Banker, G. A. The establishment of polarity by hippocampal

neurons in culture. *Journal of Neuroscience.* **8** (4), 1454-1468 (1988).

33 Arimura, N. & Kaibuchi, K. Neuronal polarity: from extracellular signals to intracellular mechanisms. *Nature Reviews: Neuroscience.* **8** (3), 194-205, doi:10.1038/nrn2056, (2007).

34 De Felice, F. G. *et al.* Alzheimer's disease-type neuronal tau hyperphosphorylation induced by A beta oligomers. *Neurobiology of Aging.* **29** (9), 1334-1347, doi:10.1016/j.neurobiolaging.2007.02.029, (2008).

35 Izuo, N. *et al.* Toxicity in Rat Primary Neurons through the Cellular Oxidative Stress Induced by the Turn Formation at Positions 22 and 23 of Aβ42. *ACS Chemical Neuroscience.* **3** (9), 674-681, doi:10.1021/cn300033k, (2012).

36 Fujiwara, H. *et al.* Uncaria rhynchophylla, a Chinese medicinal herb, has potent antiaggregation effects on Alzheimer's beta-amyloid proteins. *Journal of Neuroscience Research.* **84** (2), 427-433, doi:10.1002/jnr.20891, (2006).

37 Murakami, K. *et al.* Monoclonal antibody against the turn of the 42-residue amyloid beta-protein at positions 22 and 23. *ACS Chemical Neuroscience.* **1** (11), 747-756, doi:10.1021/cn100072e, (2010).

38 Ford, M. G. *et al.* Simultaneous binding of PtdIns(4,5)P2 and clathrin by AP180 in the nucleation of clathrin lattices on membranes. *Science.* **291** (5506), 1051-1055, doi:10.1126/science.291.5506.1051, (2001).

39 Tojima, T., Itofusa, R. & Kamiguchi, H. Asymmetric clathrin-mediated endocytosis drives repulsive growth cone guidance. *Neuron.* **66** (3), 370-377, doi:10.1016/j.neuron.2010.04.007, (2010).

40 Ahmed, G. *et al.* Draxin inhibits axonal outgrowth through the netrin receptor DCC. *Journal of Neuroscience.* **31** (39), 14018-14023, doi:10.1523/jneurosci.0943-11.2011, (2011).

41 Brennaman, L. H., Moss, M. L. & Maness, P. F. EphrinA/EphA-induced ectodomain shedding of neural cell adhesion molecule regulates growth cone repulsion through ADAM10 metalloprotease. *Journal of Neurochemistry.* **128** (2), 267-279, doi:10.1111/jnc.12468, (2014).

42 Tojima, T. *et al.* Attractive axon guidance involves asymmetric membrane transport and exocytosis in the growth cone. *Nature Neuroscience.* **10** (1), 58-66, doi:10.1038/nn1814, (2007).

43 Ooashi, N., Futatsugi, A., Yoshihara, F., Mikoshiba, K. & Kamiguchi, H. Cell adhesion molecules regulate Ca2+-mediated steering of growth cones via cyclic AMP and ryanodine receptor type 3. *Journal of Cell Biology.* **170** (7), 1159-1167, doi:10.1083/jcb.200503157, (2005).

44 Biswas, S. & Kalil, K. The Microtubule-Associated Protein Tau Mediates the Organization of Microtubules and Their Dynamic Exploration of Actin-Rich Lamellipodia and Filopodia of Cortical Growth Cones. *Journal of Neuroscience.* **38** (2), 291-307, doi:10.1523/jneurosci.2281-17.2017, (2018).

45 Kuboyama, T. *et al.* Paxillin phosphorylation counteracts proteoglycan-mediated inhibition of axon regeneration. *Experimental Neurology.* **248** 157-169, doi:10.1016/j.expneurol.2013.06.011, (2013).

Figure 1

Click here to download Figure Fig1.tif

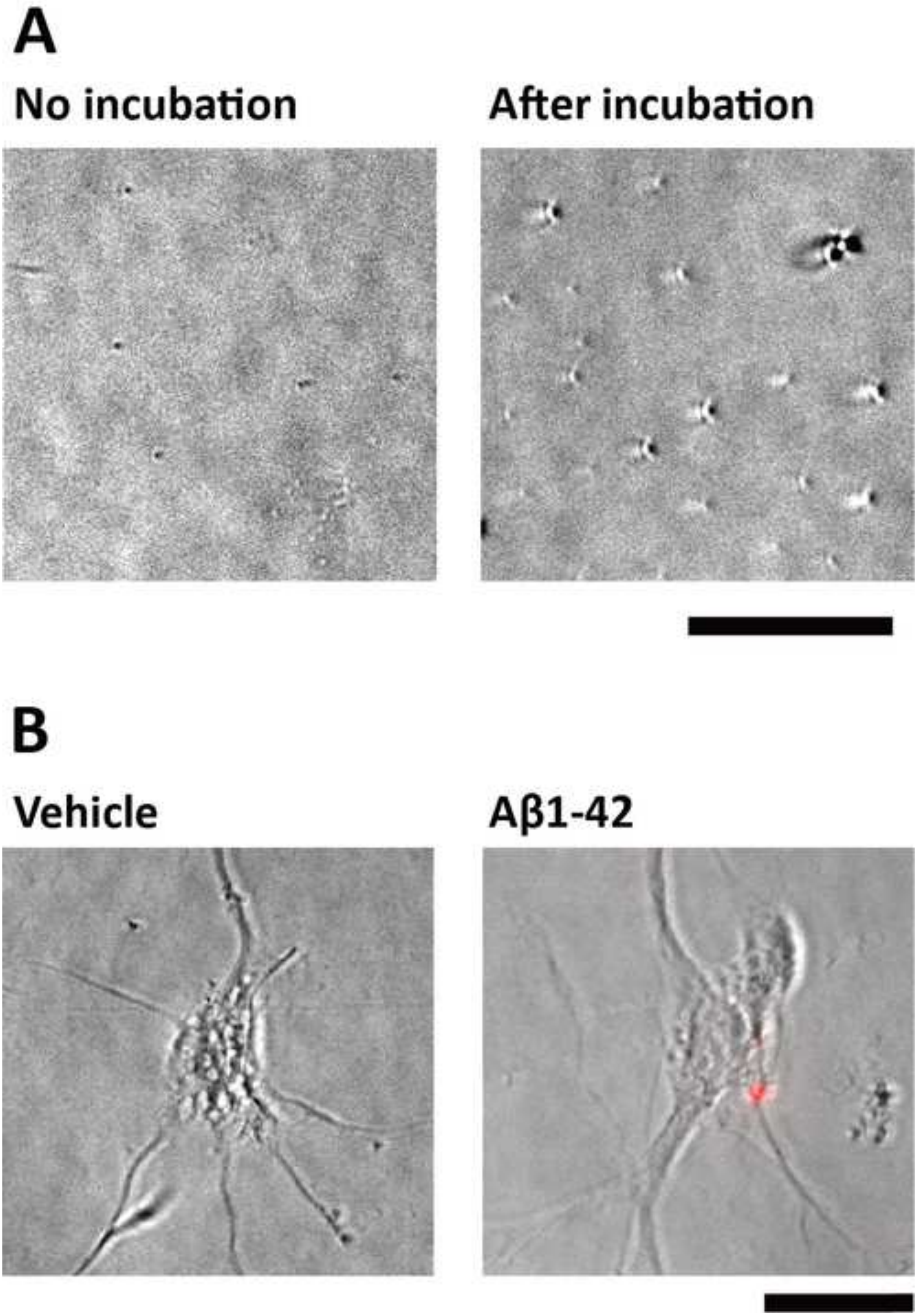


Figure 2

Click here to download Figure Fig2.tif

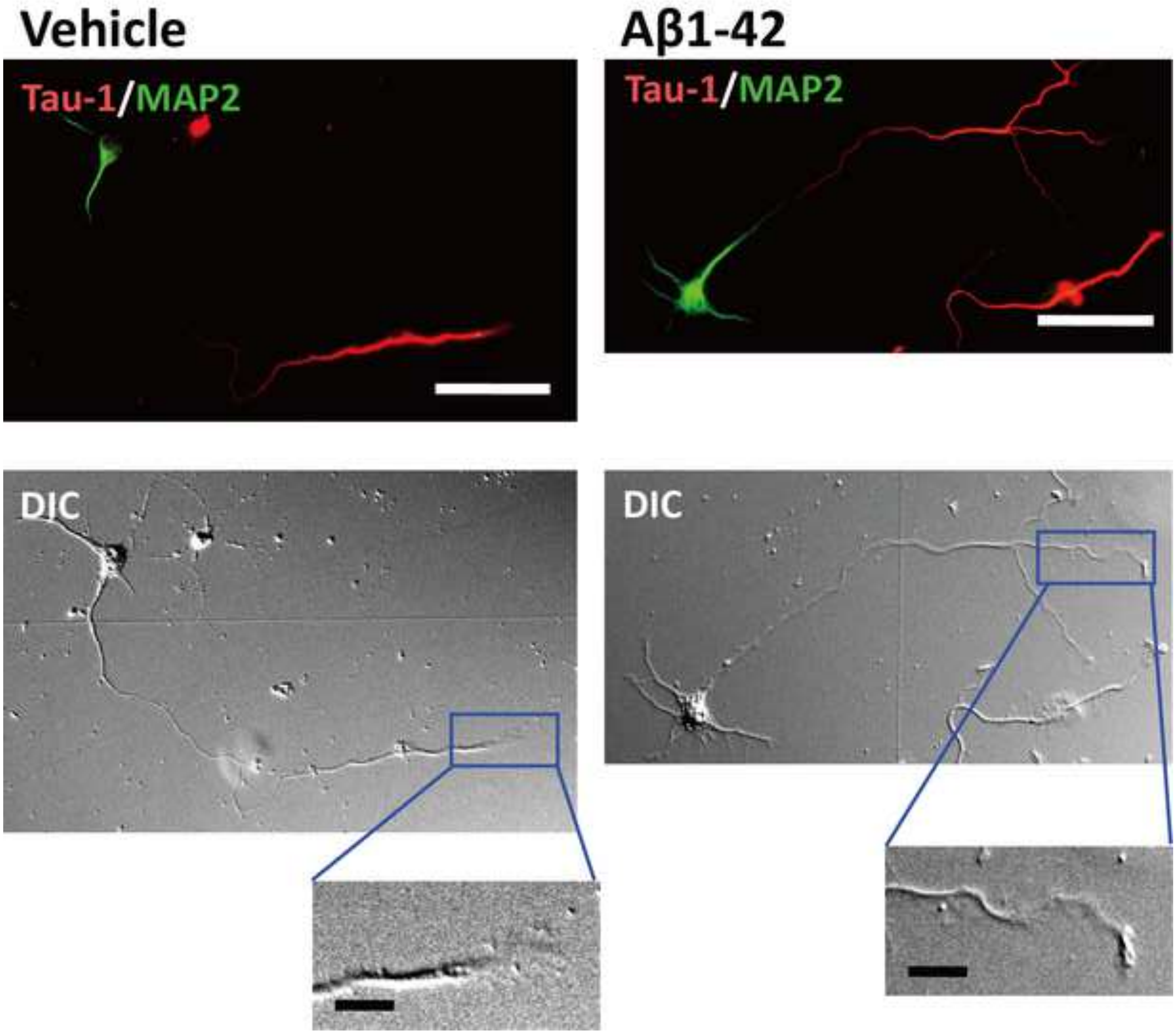
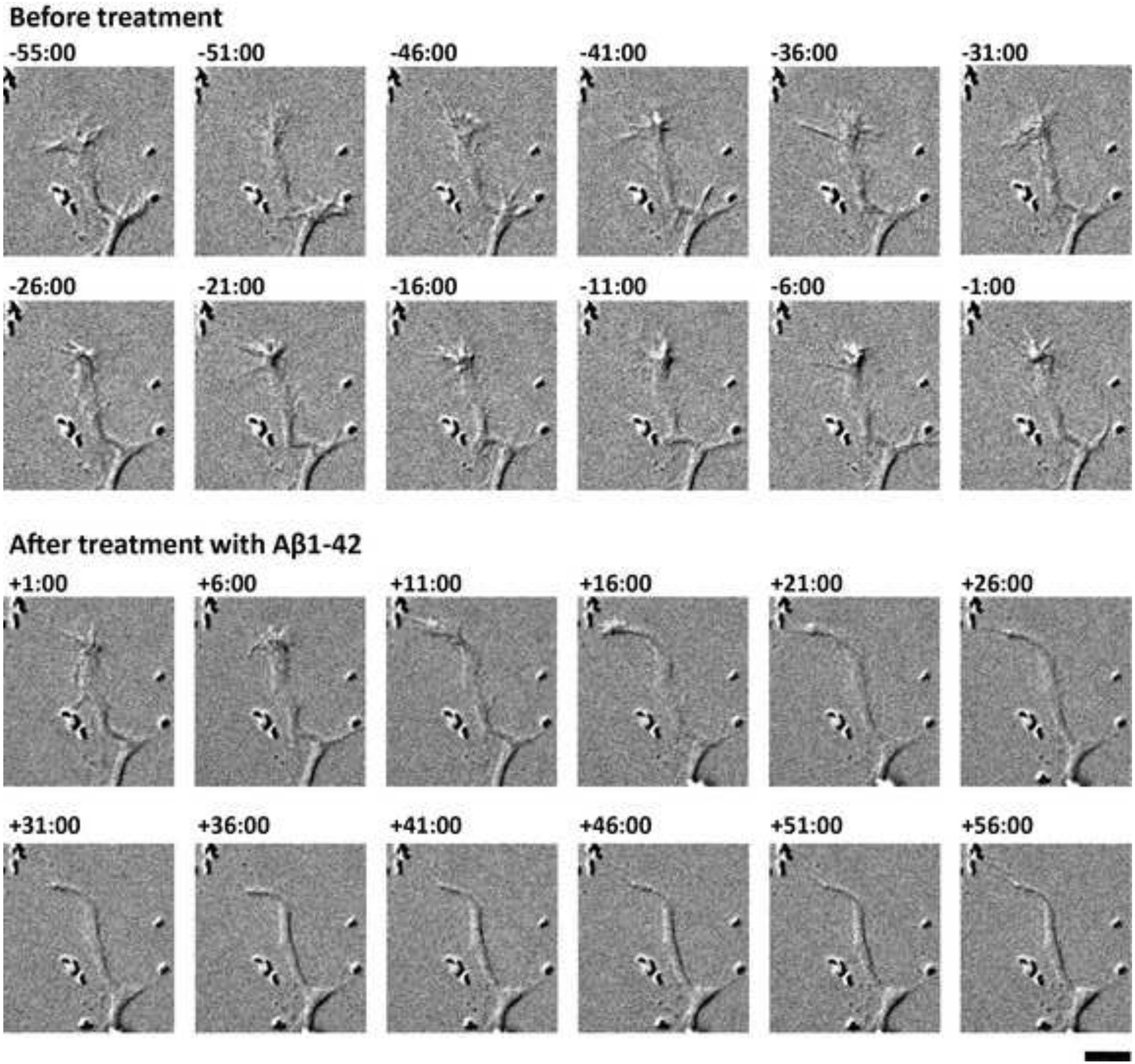


Figure 3

Click here to download Figure Fig3.tif



Click here to download Figure Fig4.tif

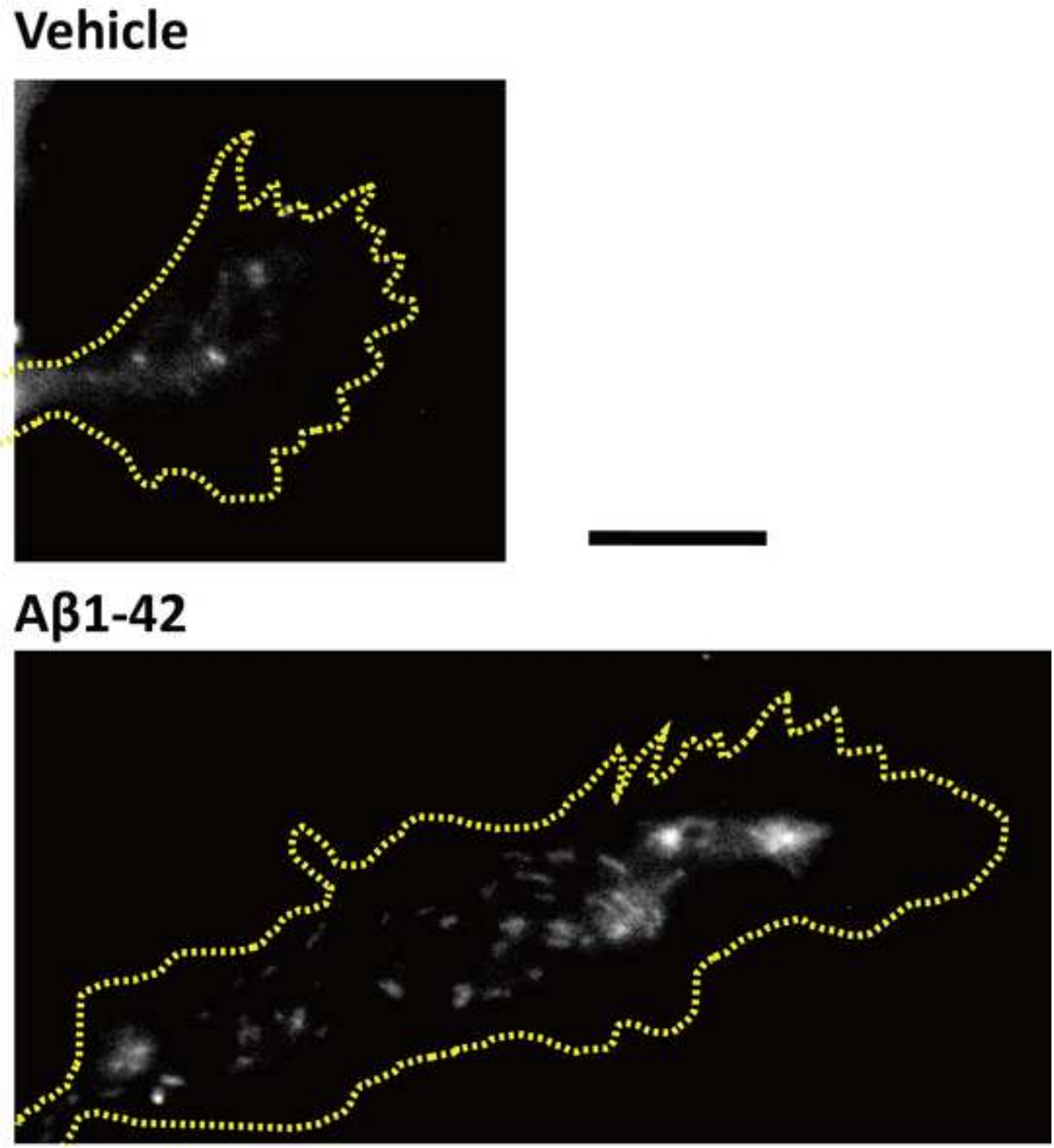


Figure 5

Click here to download Figure Fig5.tif

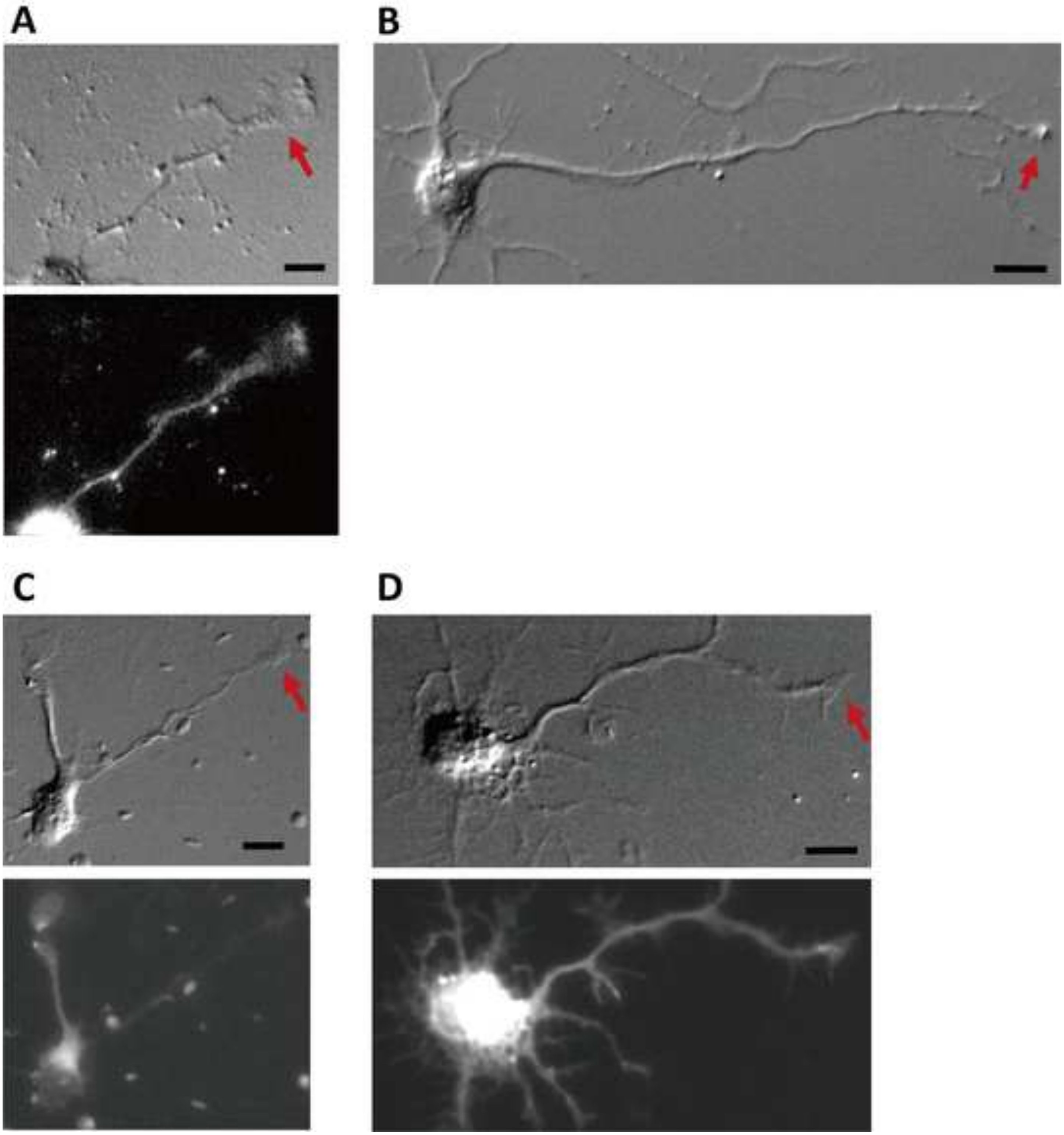


Table of Materials

Click here to download Table of Materials JoVE_Materials_kuboyama_rev.xlsx

Name of Material/ Equipment	Company	Catalog Number
ddY mice	SLC	
Eight-well culture slide	Falcon	354108
poly D lysine	Wako	168-19041
Culture medium, Neurobasal medium	Gibco	21103-049
house serum	Gibco	26050-088
glucose	Wako	049-31165
L-glutamine	Wako	074-00522
0.05% trypsin	Gibco	25300-054
DNase I	Worthington	DP
soybean trypsin inhibitor	Gibco	17075-029
Filter with 70 µm mesh size, cell strainer	Falcon	352350
B-27 supplement	Gibco	17504-044
CO_2 incubator	Astec	SCA-165DS
Amyloid β1-42	Sigma-Aldrich	A9810
paraformaldehyde	Wako	162-16065
sucrose	Wako	196-00015
Aqueous mounting medium, Aqua-Poly/Mount	polysciences	18606-20
Inverted microscope A	Carl Zeiss	Axio Observer Z1
Objective Plan-Apochromat 20x	Carl Zeiss	420650-9901
Objective Plan-Apochromat 63x	Carl Zeiss	440762-9904
Objective, CFI Plan Apo Lambda 40X	Nikon	
anti-MAP2 IgG	Abcam	ab32454

anti-tau-1 IgG	Chemicon	MAB3420
anti-amyloid β antibody	IBL	10379
normal goat serum	Wako	143-06561
bovine serum albumin	Wako	010-25783
t -octylphenoxypolyethoxyethanol	Wako	169-21105
goat anti-mouse IgG conjugated with AlexaFluor 594	Invitrogen	A11032
goat anti-rabbit IgG conjugated with AlexaFluor 488	Invitrogen	A11029
hot plate	NISSIN	NHP-M30N
cover glass	Fisher Scientific	12-545-85
35 mm dish	IWAKI	1000-035
Silicone RTV	Shin-Etsu	KE42T
hand punch	Roper Whitney	No. XX
Fluorescence membrane probe, FM1-43FX	Invitrogen	F35355
Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution	Gibco	14175-095
Transfection solution, Nucleofector solution	Lonza	VPG-1001
Electroporator, Nucleofector I	Amaxa	
Inverted microscope B	Keyence	BZ-X710
Image software, ImageJ	NIH	

Comments/Description

Connected with AxioCam MRm, Heating Unit XL S, CO_2 Module S1, and TempModule S1 clone 11A1

https://imagej.nih.gov/ij/

 Click here to download Author License Agreement (ALA) License.PDF

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Visualizing early ~~events~~ effects of amyloid β on mouse axonal growth cones

Author(s): Tomoharu Kuboyama

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: ☑ Standard Access ☐ Open Access

Item 2 (check one box):

☑ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:	Tomoharu Kuboyama
Department:	Division of Neuromedical Science
Institution:	Institute of Natural Medicine, University of Toyama
Article Title:	Visualizing early effects of amyloid β on mouse axonal growth cones
Signature:	Tomoharu Kuboyama
Date:	May 30, 2018

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Reply to the editor

Q1. The editor has formatted the manuscript as per the journal's style.

A1. I apologized that the format of the manuscript was not fit to the journal's style. Thank you very much for your efforts.

Q2. Please address all specific comments marked in the manuscript.

A2. I revised the manuscript as described in comments.

Q3. Please reword the title to better align with the highlighted text.

A3. I reworded the title as follows, Visualizing Early Effects of Amyloid β, Such as Axonal Growth Cone Collapse, in Mouse Cultured Neurons.

ELSEVIER LICENSE
TERMS AND CONDITIONS

Jul 13, 2018

This Agreement between Div of Neuromedical Sci, Instit of Natural Med, Univ of Toyama -- Tomoharu Kuboyama ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

License Number	4386911057064
License date	Jul 13, 2018
Licensed Content Publisher	Elsevier
Licensed Content Publication	Neurobiology of Aging
Licensed Content Title	Inhibition of clathrin-mediated endocytosis prevents amyloid β-induced axonal damage
Licensed Content Author	Tomoharu Kuboyama,Young-A. Lee,Hiroaki Nishiko,Chihiro Tohda
Licensed Content Date	May 1, 2015
Licensed Content Volume	36
Licensed Content Issue	5
Licensed Content Pages	12
Start Page	1808
End Page	1819
Type of Use	reuse in a journal/magazine
Requestor type	non-commercial company (non-profit)
Intended publisher of new work	other
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	electronic
Are you the author of this Elsevier article?	Yes
Will you be translating?	No
Original figure numbers	Figure 1
Title of the article	Visualizing Axonal Growth Cone Collapse and Early Amyloid β Effects in Cultured Mouse Neurons
Publication new article is in	Journal of Visualized Experiments
Publisher of the new article	MyJove Corp.
Author of new article	Tomoharu Kuboyama
Expected publication date	Sep 2018
Estimated size of new article (number of pages)	6
Requestor Location	Div of Neuromedical Sci, Instit of Natural Med, Univ of Toyama Sugitani 2630 Toyama, Toyama 9300194

	Japan Attn: Tomoharu Kuboyama
Publisher Tax ID	JP00022
Total	0.00 USD
Terms and Conditions	

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.
3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:
"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."
4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.
5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com). No modifications can be made to any Lancet figures/tables and they must be reproduced in full.
6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.
7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.
9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.
10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.
11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.
12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).
13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.
14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier

or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.

16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu. Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

17. **For journal authors:** the following clauses are applicable in addition to the above:

Preprints:

A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.). Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).

If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.
Accepted Author Manuscripts: An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.
Authors can share their accepted author manuscript:

- immediately
    - via their non-commercial person homepage or blog
    - by updating a preprint in arXiv or RePEc with the accepted manuscript
    - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
    - directly by providing copies to their students or to research collaborators for their personal use
    - for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement
- After the embargo period
    - via non-commercial hosting platforms such as their institutional repository
    - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

Published journal article (JPA): A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing, formatting, (if relevant) pagination and online enrichment.
Policies for sharing publishing journal articles differ for subscription and gold open access articles:

Subscription Articles: If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.
Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.
If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.
Gold Open Access Articles: May be shared according to the author-selected end-user license and should contain a CrossMark logo, the end user license, and a DOI link to the formal publication on ScienceDirect.
Please refer to Elsevier's posting policy for further information.
18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.
19. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

Elsevier Open Access Terms and Conditions

You can publish open access with Elsevier in hundreds of open access journals or in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author's choice of Creative Commons user license. See our open access license policy for more information.
Terms & Conditions applicable to all Open Access articles published with Elsevier:

Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation. If any changes have been made, such changes must be clearly indicated.
The author(s) must be appropriately credited and we ask that you include the end user license and a DOI link to the formal publication on ScienceDirect.
If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.
Additional Terms & Conditions applicable to each Creative Commons user license:
CC BY: The CC-BY license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by/4.0.
CC BY NC SA: The CC BY-NC-SA license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. Further, any new works must be made available on the same conditions. The full details of the license are available at http://creativecommons.org/licenses/by-nc-sa/4.0.
CC BY NC ND: The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by-nc-nd/4.0. Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee.
Commercial reuse includes:

- Associating advertising with the full text of the Article

- Charging fees for document delivery or access
- Article aggregation
- Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

20. **Other Conditions**:

v1.9

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.