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Establishment of a valuable mimic Alzheimer's disease animal model by intracerebroventricular injection of composited amyloid beta protein

--Manuscript Draft--

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Abstract:	SHORT ABSTRACT: This manuscript describes a detail protocol for a mimic AD animal model by evaluation of spatial memory impairment, neuronal pathological changes, neuronal amyloid beta protein (Aβ) burden and neurofibrillary tangles (NFT) aggregation induced by injection of Aβ25-35 combined with aluminum trichloride (AlCl3) and recombinant human transforming growth factor-β1 (RHTGF- β1) in rats. LONG ABSTRACT: Alzheimer's disease (AD) is an irreversible, progressive brain disease that slowly destroys memory and accompanied by neurons loss and structure change. Along with the increase of AD patients in worldwide, the effort of the pathology and treatment for the disease has become a focus in the International Pharmaceutical Industry. Then, the establishment of the animal model to mimic AD is of great importance in laboratory. In the present video, we described a detailed protocol for establishing mimic AD animal model though intracerebroventricular injection of Aβ25-35 combined with AlCl3 and anterodorsal thalamic nucleus injection of RHTGF- β1 to rats. The related markers of AD including spatial memory, neuronal structure/substructure, neuronal Aβ and NFT production were measured. This result was observed that these states spatial memory impairment, neuronal structure/substructure pathological changes, and neuron intracellular Aβ burden and NFT aggregation in this model rats. These neuronal structure and function disorders are the closest to the status of clinical AD patients. Then, the present rats' model provides a valuable in vivo animal model for exploring neuronal function and neuronal pathology and drug screening of AD.
Author Comments:	Auyhors carefully revised the manuscript according to editor and reviewers suggestion, and supplement other data related to AD.

Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date, please indicate the date below and explain in your cover letter.	

June 20th, 2017

Dear editors of *JoVE:*

Please find enclosed a manuscript entitled “Establishment of a valuable mimic Alzheimer’s disease animal model by intracerebroventricular injection of composited amyloid beta protein” that we wish to submit as an visualized article to *Journal of Visualized Experiments*.

It is well known that AD is a neurodegentive disease accompanied by multifactor participated. Aβ25-35 is a neurotoxin, and can result in lots of neuron functional disorders and injuries. Then, the imitated AD model in animal with multi-factor neuro-damages established from Aβ25-35 in combination with aluminum trichloride (AlCl3) and recombinant human transforming growth factor-β1 (RHTGF-β1) (composited Aβ) was the more comprehensive simulation in histopathogenic traits of human. Aluminum can prevent the sedimentary Aβ into soluble Aβ, and RHTGF-β1 can also boost the sedimentary Aβ formation and accelerate AD occurrence. Here, in the present study, the Aβ25-35 in combination with AlCl3 and RHTGF-β1 were intracerebroventricularly injected to rats for building a model of Alzheimer’s-disease-like memory impairment and brain injuries were reported using this model.

Morris water maze was used to screen the successful model of rats’ spatial memory impairment. The successful rate of memory impairment model in the present study was 94.7%. In the determination of rats memory with Morris water maze task, the model group always took longer latencies to find the hidden platform in positioning navigation trial and reversal trial, and spent shorter time swam in target quadrant (the first quadrant, Q1) in probe trial. The optical, light and electron microscopic observations found significant neuropathological changes including cortical injuries, neuron loss/swelling, neuron Aβ burden/NFT aggregation and other neuronal damages.

All authors have approved the manuscript and have agreed to submit to *JoVE*. Please find enclosed the manuscript, figures and supplement. In addition, the present manuscript was based on our lab previous published paper and focus on the animal model established. The previous published paper has been cited but re-used data and figures. We would like submit the manuscript to JoVE and is not under consideration by another journal. If you need any additional information, please feel free to contact me. We look forward to hearing from you.

Sincerely,

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TITLE:
Establishment of a Valuable Mimic of Alzheimer's Disease in Rat Animal Model by Intracerebroventricular Injection of Composited Amyloid Beta Protein

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KEYWORDS:
Amyloid beta protein 25-35, aluminum trichloride, recombinant human transforming growth factor-β1, composited Aβ, Alzheimer's disease model, memory impairment, neuropathology, amyloid beta protein burden, neurofibrillary tangles aggregation

SHORT ABSTRACT:
This is a protocol to mimic Alzheimer's Disease in rats by evaluation of spatial memory impairment, neuronal pathological changes, neuronal amyloid beta protein (Aβ) burden, and neurofibrillary tangles aggregation, induced by the injection of Aβ25-35 combined with aluminum trichloride and recombinant human transforming growth factor-β1.

LONG ABSTRACT:
Alzheimer's disease (AD) is an irreversible, progressive brain disease that slowly destroys memory and is accompanied by neuron loss and structure change. With the increase of AD patients worldwide, the pathology and treatment of the disease has become a focus in the International Pharmaceutical Industry. Thus, the establishment of the animal model to mimic AD in the laboratory is of great importance.

In the present video, we describe a detailed protocol for establishing a mimic of AD in a rat animal model though intracerebroventricular injection of amyloid beta protein 25-35 (Aβ25-35) combined with aluminum trichloride ($AlCl_3$) and anterodorsal thalamic nucleus injection of recombinant human transforming growth factor-β1 (RHTGF-β1) to rats. The related markers of AD were measured, including: spatial memory, neuronal structure and substructure, neuronal Aβ,

and neurofibrillary tangles (NFT) production. This rat model demonstrates spatial memory impairment, neuronal structure and substructure pathological changes, neuron intracellular Aβ burden, and NFT aggregation, and provides a close mimic of the neuronal structure and function disorder to that of clinical AD patients. Thus, the presented AD rat model provides a valuable *in vivo* tool for exploring neuronal function, neuronal pathology, and drug screening of AD.

INTRODUCTION:

It is well known that AD is a chronic and progressive neurodegenerative disease, with gradual memory loss as the main clinical syndrome. In the general pathology, there is nervous tissue atrophy, neuron and synapse loss, as well as neuronal subcellular structure and function disorders, which are all involved in the development and clinical manifestation of AD[1,2]. It is reported that when animals were intracerebroventricularly injected with Aβ, some neurotoxic events occur in the brain involving neuron loss, calcium homeostasis disruption, neuron apoptosis, and reactive oxygen species overproduction[3]. However, multiple factors are involved in the pathogenesis of AD and thus it is essential to establish a better model of AD.

A detailed protocol is described here for establishing an *in vivo* mimic AD model through intracerebroventricular injection of Aβ25-35 and $AlCl_3$, combined with anterodorsal thalamic nucleus injection of RHTGF-β1 to rats. This rat model highly mimics human neuronal function and histopathogenesis of AD, including memory impairment, neuron loss and structure damage, apoptosis, intracellular Aβ burden, and NFT aggregation[4-9]. The $AlCl_3$ prevents the deposited Aβ from forming soluble Aβ, and the RHTGF-β1 can promote deposited Aβ production and facilitate AD occurrence[10]. This attack from several factors to the neuron is in accordance with the multi-pathogenesis of AD.

The entire experiment spanned 86 days: **Figure 1** shows a timeline of the experimental design, with the time point of animal surgery, animal model screening, animal spatial memory test, and sample preparation. On the first day of operation, RHTGF-β1 was microinjected into the anterodorsal thalamic nucleus. On the second day of operation, Aβ25-35 and $AlCl_3$ were microinjected into the lateral ventricle daily for 14 consecutive days in the morning and 5 consecutive days in the afternoon, respectively. All rats were allowed to recover for 45 days after the operation. The Morris water maze was used to screen for animal model rats with memory impairment and to assess their spatial memory. The rats underwent 4 consecutive days of water maze training with 2 trials per day, and on day 4 of training, the rats were evaluated with the Morris water maze for memory impairment. All rats continued to be fed 37 days after the animal model screening. The spatial memory of rats was tested in the Morris water maze over 7 consecutive days, from day 79 to day 85 after the operation. All rats were sacrificed by decapitation on day 86 for brain sample preparation.

Insert Figure 1 here

PROTOCOL:

This procedure was in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of China on Oct. 31, 1988[11]. Scientists should follow the guidelines established and approved by their institutional and national animal regulatory organizations.

Note: Animal and regents: Four-month-old male Sprague-Dawley rats (300–350 g) were supplied for this experiment. All rats were housed in groups (four or five per cage) at a temperature of 23 ± 1 °C with a 12-h light-dark cycle. Food and water were available *ad libitum*. The rats were acclimatized to the housing conditions for 7 days before the procedure was performed. Aβ25-35 was dissolved in 1% DMSO saline to 1 mg/mL, and sonicated for 5 min in an ultrasonic oscillator until completely dissolved. $AlCl_3$ and RHTGF-β1 were dissolved in saline at 1% and 0.1 mg/mL, respectively. Congo red, silver nitrate, and other chemicals were of analytical grade and were purchased from ordinary commercial sources.

1. Surgical Procedure

Note: 20 male Sprague-Dawley rats were microinjected with composited Aβ into the lateral ventricle and anterodorsal thalamic nucleus, and designated as the composited Aβ-treated group. Another 20 rats were subjected to the same operation but received 0.1% DMSO saline microinjection, and designated as the sham-operated group.

1.1. Anaesthetize the rats with 100% isoflurane by inhalation and then restrain on a Brain Stereotaxic Apparatus.

1.2. Snip the rat's hair on the head vertex with surgical scissors and disinfect with iodophor.

1.3. Make an incision on the head skin along the median longitudinal calvaria with surgical bistouries and scissors.

1.4. Separate the subcutaneous tissue and fascia, wipe the skull calvarium with 0.3% H_2O_2, and mark the bregma with a marker pen.

1.5. Refer to the Rat Brain Stereotaxic Map[12]; considering the bregma as the point of origin, gently drill a 0.1 mm diameter hole vertical to the skull (posterior (P): 2.0 mm to the bregma; lateral (L): 1.4 mm to the midline; and anterodorsal thalamic nucleus (ad): 4.6 mm to the skull) with a flexible bone drill (**Supplemental File 1**).

1.6. Insert a microinjector to the brain at 4.6 mm depth and gently inject 1 μL RHTGF-β1 (10 ng) into the ad area.

1.7. Fix the inserted needle point into lateral ventricle (LV) area (posterior (P): 0.8 mm to the bregma; lateral (L): 2.0 mm to the midline; and ventral (V): 4.6 mm to the skull) (**Supplemental File 2**).

1.8. Gently drill a 0.2 mm diameter hole with flexible bone drill and remove the skull surface to expose the endocranium under the bone plate.

1.9. Stop the bleeding with medical bone wax and clean the skull surface repeatedly with sterile dry cotton.

1.10. Assemble the cannula implantation system (**Supplemental File 1**) after disinfection with 75% alcohol immersion for 24 h.

1.11. Insert the stainless-steel tubing guide cannula to the brain through the skull hole.

1.12. Drill 2 holes with diameter 0.2 mm on the skull and turn the screw to lock the thread of guide cannula plastic pedestal. Do not screw deeper than necessary to avoid stabbing the brain tissue.

1.13. Mix the zinc phosphate dental cement with water at ratio of 1 g:0.5 mL, and put the paste around the guide cannula plastic pedestal. After the paste dries, draw out the dummy cannula, and observe that clear cerebrospinal fluid outflows from the upper guide cannula to ensure placement of the guide cannula in the LV.

1.14. Insert the dummy cannula in the guide cannula, disinfect, and stitch the wound.

1.15. On day 2 of the operation, anaesthetize the rats with isoflurane inhalation using the Small Animal Anesthesia Machine. Draw out the dummy cannula, insert the internal cannula into the guide cannula, and screw the fixing screw to immobilize the internal cannula.

1.16. Set the polyethylene pipe that links the microinjection pump to the internal cannula and regulate the injection speed to 1 μL/min. Microinject the Aβ25-35 or $AlCl_3$ to the LV.

1.17. Microinject 4 μg (1 μL) Aβ25-35 daily for 14 days in the morning and 3 μL $AlCl_3$ (1%) daily for 5 days in the afternoon under isoflurane anesthesia.

1.18. Wait 5 min after finishing the injection, gently draw out the **internal cannula and** insert the dummy cannula again into the guide cannula.

1.19. On day 15 post surgery (which corresponds to the last injection day of Aβ25-35) dismantle the cannula implantation system. Gently remove the zinc phosphate dental cement with Rongeur and hemostatic forceps, unscrew the screws, pull out the guide cannula, and disinfect the wound with betadine. Fill in the hole of the skull with bone cement and suture the skin with a simple interrupted suture method.

1.20. Perform the same operation with the sham-operated group and microinject 0.1% DMSO saline.

1.21. Postoperative nursing

1.21.1. Inject penicillin 10 U/kg body weight to all operated rats by intraperitoneal injection for 3 days after operation to prevent infection.

1.21.2. House 2 rats per cage after operation and provide food for 30 days.

Note: 18 rats in the sham-operated group survived (90% success rate of operation), and 19 rats in

the composited-treated Aβ group survived (95% success rate of operation).

2. Screening for Successful Model Rats and Assessment of Spatial Memory with Morris Water Maze

2.1. Morris water maze

Note: The Morris water maze was used to assess rat spatial memory[13]. The Morris water maze is a stainless-steel circular tank with 120 cm diameter and 50 cm depth. The water maze test was performed, based on the "gold standards" paradigm described in Behavioral Neuroscience by J. Nunez[14].

2.1.1. Blacken the pool water with several drops of ink.

2.1.2. Maintain the depth of water at 31.5 cm and the temperature at 23 ± 1 °C.

2.1.3. Set a 1.5 cm circular transparent plexiglass platform below the water surface.

2.1.4. Maintain that all spatial signals around the water maze are invariable during the water maze tests.

2.1.5. Divide the pool into 4 equal quadrants by imaginary lines for descriptive data collection.

2.1.6. Place the hidden platform in the first quadrant (Q1) of water maze.

2.1.7. Capture the rat swimming behaviors (measured by latency, trajectory, or crossing number) through a video camera, over the water maze linked to a computer-based graphics analytic software.

2.2. Screening for successful model rats to the composited Aβ-treated group

2.2.1. On day 45 of surgery, perform the Morris water maze training for 4 consecutive days to screen for successful model rats with memory impairment and collect the screening ratio (SR).

Note: The SR is defined as the average latency of each composited Aβ-treated rat and the sham-operated group of rats to find the hidden platform under the water surface on day 4 of water maze training. “A” is the average latency of each composited Aβ-treated rat to find the hidden platform and “B” is the average latency of the sham-operated group of rats to find the hidden platform, on day 4 of water maze training, in the following equation:

$$SR = \frac{A - B}{B}$$

When SR was larger than 0.2 for one composited Aβ-treated rat, the rat was regarded as a successful model rat with impaired memory of composited Aβ-treated rat[15]. The intraday memory performance of rats was determined by the data of 2 trials of rats for the average time to find the hidden platform. The procedure of the Morris water maze test was designed such that the

rats were allowed to swim and look for the hidden platform within 60 s. If a rat did not find the hidden platform within 60 s, the rat then was placed on the platform by the experimenter. When a rat reached the hidden platform (independently or assisted), the rat was permitted to remain there for 20 s. Then, the rat was removed from the pool and allowed a physical recovery for 10 s between the 2 trials.

2.3. Determination of rat spatial memory

2.3.1. Evaluate the spatial memory of rats for 7 consecutive days with 2 trials every day using the Morris water maze test.

Note: The measurement of spatial memory was divided into 4 parts: 2 days of positioning navigation trial for memory acquisition, 1 day of probe trial for memory retention, 3 days of reversal trial for memory re-learning, and finally 1 day of visible platform trial for swimming speed.

Note: Positioning of the navigation trial and reversal trial: The positioning navigation trial was used to calculate memory acquisition on day 1–2 of the Morris water maze test, which was carried out on day 79 and 80 after the operation (**Figure 1**). The location of the hidden platform was the same as in the model screening (Q1). The average value of latency over 2 trials was taken as the intraday memory acquisition achievement. 3 consecutive test days on day 4, 5, and 6 of the reversal trial were carried out and the rat memory re-learning was estimated, which corresponded to day 82, 83, and 84 after the operation (**Figure 1**). The platform was set on the opposite side of the target quadrant (Q3). The average latency over 2 trials was recorded as the rat intraday re-learning performance.

Note: Probe trial: The probe trial was used to calculate the rat memory retention on day 3 of the Morris water maze test, which corresponded to day 81 after the operation (**Figure 1**). The platform was removed from the pool, and the rats were permitted to swim for 60 s to look for the target quadrant (Q1) where the platform was previously placed in the positioning navigation trial. Swimming time, swimming distance, and crossing number within the target quadrant (Q1) for 60 s of the rat were recorded and taken as the memory retention performance.

2.3.2. Perform the visible platform trial to exclude the influence of motivational or sensorimotor factors on learning and memory performance, and to estimate the rat swimming speed on day 7 of the Morris water maze test.

Note: The platform was 2 cm over the water surface and the visible platform trial was conducted for 1 day on day 85 post surgery (**Figure 1**). The rat swimming path length and time to step on the platform were recorded and calculated as the rat swimming speed.

3. Neuron examination

3.1. On day 86 post surgery, under ether anesthesia, sacrifice the rats by decapitation (**Figure 1**).

3.2. Put the brain on ice and gently separate the two hemispheres at the raphe. Take the left

hemisphere of the optic chiasma and fix in 4% formaldehyde for light microcopy observation of neuron hematoxylin and eosin (HE), Congo red, or silver nitrate stain (see Sections 4–6). Fix the right hemisphere hippocampus CA1 area in 2.5% glutaraldehyde for electron microcopy observation (Section 7).

3.3 Process the brain for the light/electron microcopy sample preparation, as previously described[16,17].

4. Neuron HE Staining

4.1. Deparaffinize each slide (20 min each) with gradient alcohol (100%, 95%, 90%, 80%, and 70% alcohol) to distilled water in a fume hood.

4.2. Stain for 3 min with hematoxylin (0.5% w/v), and then rinse with tap water to remove the unbound dye from slides.

4.3. Rinse with 0.1% hydrochloric acid in alcohol for 1 s to remove the color of unstained nuclei.

4.4. Immerse in 0.5% ammonia solution for 2 min until the background turns light blue.

4.5. Stain for 1 min with 1% eosin.

4.6. Dehydrate for 5 min with gradient alcohol (70%, 80%, 90%, 95%, and 100% alcohol).

4.7. Clear in xylene and mount with resinous mounting medium.

4.8. Observe and count the living neurons of the HE stain per 0.125 mm in the middle CA1 of the hippocampus and per 0.0352 mm^2 in the cerebral cortex at a magnification of 400x with an optical microscope by a person blinded to the experimental design.

5. Congo Red Staining for Assaying Neuron Aβ Burden

5.1. Deparaffinize each slide (20 min) with gradient alcohol (100%, 95%, 90%, 80%, and 70% alcohol) to distilled water in a fume hood.

5.2. Stain for 20 min with Congo red working solution (0.5 g Congo red, 80 mL methyl alcohol, 20 mL glycerinum).

5.3. Rinse in distilled water for 5 min.

5.4. Differentiate quickly with alkaline 80% alcohol solution (0.2 g alcohol/100 mL potassium hydroxide) for 3 s.

5.5. Rinse twice, each for 5 min with distilled water.

5.6. Counterstain in Gill's hematoxylin for 3 min.

5.7. Rinse in tap water for 2 min.

5.8. Dip in ammonia water (add a few drops of ammonium hydroxide to tap water and mix well) for 30 s or until the sections turn blue.

5.9. Rinse in tap water for 5 min.

5.10. Dehydrate with gradient alcohol (70%, 80%, 90%, 95%, and 100% alcohol).

5.11. Clear in xylene and mount with resinous mounting medium.

5.12. Observe and count the cells stained with Congo red at a magnification of 400x, with an optical microscope by a person blinded to the experimental design.

6. Silver Nitrate Staining for Assaying Neuron NFT Formation

6.1. Deparaffinize each slide (20 min) with gradient alcohol (100%, 95%, 90%, 80%, and 70% alcohol) to distilled water in a fume hood.

6.2. Immerse in 20% silver nitrate solution and capping agent for 20 min in the dark.

6.3. Wash in distilled water twice, 5 min for each time.

6.4. Immerse in silver ammonia solution. Drop ammonia solution into 20% silver nitrate solution, and add until the solution goes from turbidity to clarification. Simultaneously, stir the solution with a glass rod for 15 min and then put into the 1% diluted ammonium hydroxide for 2 min.

6.5. Place the slide into the developing working solution for 3–7 min until the black block in the axon can be observed.

6.6. Immerse in the 0.1% diluted ammonia solution for 1 min and then rinse with water for 1 min.

6.7. Dispose with 5% sodium thiosulfate for 2 min and then rinse with water for 5 min.

6.8. Dehydrate with gradient alcohol (70%, 80%, 90%, 95%, and 100% alcohol).

6.9. Clear in xylene and mount with resinous mounting medium.

6.10. Observe and record the cell for silver nitrate stain at a magnification of 400x with an optical microscope by a person blinded to the experimental design.

7. Hippocampal Neuron Ultrastructure Measurement

7.1. Cut the rat hippocampus CA1 into several cubes (1 x 1 x 1 mm^3) and place in 2.5% glutaraldehyde for 2 h at 4 °C.

7.2. Rinse the cubes with PBS three times (pH 7.2, 10 min for each time).

7.3. Fix the cubes in 1% osmic acid for 2 h at 4 °C.

7.4. Rinse the cubes in double distilled water 3x (10 min for each time).

7.5. Dehydrate with gradient alcohol 50%, 70%, and 90% (10 min for each), 100% two times (15 min for each time).

7.6. Replace by propylene oxide two times (15 min for each time), propylene oxide:resin at 1:1 (60 min for each time at room temperature), and propylene oxide:resin 1:4 (60 min for each time at room temperature). Soak in resin (120 min, at room temperature).

7.7. Embed in EPON 812 and polymerize (5 h/35 °C, 5 h/60 °C, 5 h/80 °C).

7.8. Cut the semithin sections (1 μm), stain by methylene blue, and localize under the microscope.

7.9. Stain the ultra-thin sections (50 nm) with uranyl-o-acetate and lead citrate.

7.10. Examine under a JEM-1400 electron microscope and collect images.

REPRESENTATIVE RESULTS:

All data are presented as mean ± SEM. SAS/STAT package was used to perform the statistical analysis. The group differences in latency to find the hidden platform in the Morris water maze test were analyzed by two-way analysis of variance (ANOVA) with repeated measures. The group differences in the probe trial and number of neurons were analyzed by one-way ANOVA followed by Duncan's multiple-range test. $p < 0.05$ was considered statistically significant.

Screening for Successful Model Rats with Memory Impairment for the Composited Aβ-treated Group:

The results in **Figure 2AA1 and 2AA2** show that the sham-operated group of rats always swam freely and the composited Aβ-treated group rats (**Figure 2AB1, AB2**) always swam around the pool perimeter in adaptive swimming in the Morris water maze. Over the 4 days of screening for memory impairment model rats, all rats had progressively declining times to find the hidden platform (latency) (**Figure 2B**). On day 4 of Morris water maze training, if the SR was more than 0.2 (which was based on the latency of each composited Aβ-treated rat and the sham-operated group of rats for finding the hidden platform), than the composited Aβ-treated rat was considered a successful model rat with memory impairment. 18 of the 19 rats (94.70%) that survived the operation passed the successful model screening. 6 rats of each group were chosen for the following experiments.

Insert Figure 2 here

Composited Aβ Caused Rat Memory Acquisition and Memory Re-learning Impairments:

The rat memory acquisition was determined by the positioning navigation trial on day 1 and 2 of the Morris water maze test, which corresponded to day 79 and 80 post surgery. During the 2 days of the memory acquisition trial, all rats exhibited progressively declining latency to find the hidden platform. As shown in **Figure 3**, the latencies of the composited Aβ-treated group for finding the hidden platform were 360.67% and 558.28% (F (1, 5) = 238.67, $p < 0.01$) greater than those of the sham-operated group on day 1 and 2 of the Morris water maze test, respectively. This indicates that the composited Aβ can induce memory acquisition impairment in rats.

The rat memory re-learning was assayed by the reversal trial on day 4, 5, and 6 of the Morris water maze test, which corresponded to day 82, 83, and 84 post surgery. As shown in **Figure 3**, the latencies of the composited Aβ-treated group for finding the hidden platform were 306.20%, 650.16%, and 936.92% longer time than those of the sham-operated group (F (1, 5) = 138.76, $p < 0.01$). This demonstrates that the composited Aβ can elevate the memory re-learning impairment in rats (**Figure 3**).

Insert Figure 3 here

Composited Aβ Caused Rat Memory Retention Impairment:

The rat memory retention was measured by probe trial on day 3 of the Morris water maze test, which corresponded to day 81 post surgery. In the 1 day memory retention trial, the composited Aβ-treated group took less swimming time, swimming distance, and crossing number in Q1 within 60 s, which corresponded to 32.14%, 30.11%, and 78.95% ($p < 0.01$), respectively, than those of the sham-operated group (**Figure 4A, 4B**). These results show that the composited Aβ can produce memory retention impairment in rats.

Insert Figure 4A and 4B here

Composited Aβ Influenced Rat Swimming Speed:

The rat swimming speed was calculated by the visible platform trial on the day 7 of Morris water maze test, which corresponded to day 85 post surgery. The rat swimming speed, based on the calculation of swimming distance and time to step on the platform, of each group in the pool was not significantly different. Therefore, the individual differences in rat swimming speed could be excluded, which indicates that motivation and motor skills were essentially intact in all rats (**Figure 5**).

Insert Figure 5 here

Composited Aβ Caused Rat Neuronal Morphological Change:

All rats were decapitated on day 86 post surgery. A yellow surface and a thin or collapsed cerebral cortex were observed in several composited Aβ-treated rats by visual inspection. Optical microscopy of HE stained brains from the composited Aβ-treated group showed marked pathological changes in neurons of the hippocampus, such as neurofibrillary degeneration, neuronophagia, nuclear pyknosis, and nuclear margination (**Figure 6AB2**), as compared with the sham-operated group (**Figure 6AA2**). In addition, neurons in part of the cerebral cortex of the composited Aβ-treated rats showed typical colliquative necrosis, which was characterized by disrupted cell membranes, fragmented nuclei, and extensive infiltration of inflammatory cells in the necrotic region (**Figure 6AB3**). This indicates that the composited Aβ can cause neuronal pathological injuries in rats.

Insert Figure 6A here

In addition to pathological changes, the neuron number was also significantly reduced in the hippocampus and cerebral cortex (except for the colliquative necrosis sample) in the composited Aβ-treated group, as compared with the sham-operated group. The neuron count was 63.86% ($p < 0.01$) lower than that of the sham-operated group in 0.125 mm hippocampal CA1 sections, and 55.46% ($p < 0.01$) lower in 0.0352 mm^2 cerebral cortex sections (**Figure 6B**), which suggests that the composited Aβ can result in a lower neuron count.

Insert Figure 6B here

The ultrastructure of the hippocampus neurons was observed with electron microscopy. Compared with the sham-operated group (**Figure 7A1–A4**), neurons in the composited Aβ-treated group (**Figure 7B1–B4**) were severely injured in substructure, showing mitochondrial swelling and cristae fragmentation, increased mitochondrial electron density, dilated rough endoplasmic reticulum, depolymerized polyribosomes and polymicrotubules, some postsynaptic density (PSD), many secondary lysosomes, and lipofuscin deposits in cytoplasm. The nuclear membrane appeared rough and sunken, the euchromatin was condensed and denatured, the myelin sheath layers were loose or attenuated, and internal axons and fibers were degenerated. These results demonstrate that the composited Aβ can produce neuron sub-structure damage in rats.

Insert Figure 7 here

Composited Aβ Caused Aβ Burden in Rat Neurons:

Congo red staining was used to detect the Aβ burden on neurons. The results show that the composited Aβ can notably induce the intracellular Aβ burden in the rat hippocampus and cerebral cortex (**Figure 8A**). The positive number of cells with Aβ red stained by Congo red in the hippocampus and cerebral cortex of the composited Aβ-treated group are 8.05- and 4.09-fold ($p < 0.01$) greater than those of the sham-operated group (**Figure 8B**). This demonstrates that composited Aβ can increase neuron Aβ burden in rats.

Insert Figure 8A and 8B here

Composited Aβ Caused NFT Deposition in Rat Neurons:

Silver nitrate staining was used for detecting the NFT deposition in neurons. The results show that composited Aβ can noticeably cause the intracellular NFT deposition in the rat hippocampus and cerebral cortex (**Figure 9A**). The positive number of cells with NFT brown stained by silver nitrate in the hippocampus and cerebral cortex of the composited Aβ-treated group are 9.75- and 4.82-fold ($p < 0.01$) greater than those of the sham-operated group (**Figure 9B**). This demonstrates that the composited Aβ can increase the neuron NFT aggregation in rats.

Insert Figure 9A and 9B here

FIGURE LEGENDS:
Figure 1. The timeline of the experimental design.

Figure 2. Screening for successful model rats with memory impairment in the composited Aβ-treated group using the Morris water maze training. (**A**) The adaptive swimming trajectory of rats in the Morris water maze. (**AA1–AA2**) Sham-operated group; (**AB1–AB2**) Composited Aβ-treated group. (**B**) Mean latency to find the hidden platform for 4 consecutive days of the screening trial in the Morris water maze training for the sham-operated group and the composited Aβ-treated group.

Figure 3. Composited Aβ caused rat memory acquisition and memory re-learning impairments. The positioning navigation trial was used to evaluate memory acquisition by 2 consecutive days swimming achievement on day 1 and 2 in the Morris water maze test. These were performed on day 79 and 80 post surgery. The reversal trial was used to evaluate memory re-learning by 3 consecutive days swimming score on day 4, 5, and 6 in the Morris water maze test, which corresponded to day 82, 83, and 84 of the operation. The line graph plots show the mean latency to find the hidden platform for each group on day 1, 2, 4, 5, and 6 in the Morris water maze test. Data were analyzed by two-way ANOVA (day x group) with repeated measures. Mean ± SEM. n = 6. $^{**}p < 0.01$, vs. Sham-operated group.

Figure 4. Composited Aβ produced rat memory retention impairment. The probe trial was used to evaluate memory retention by 1 day swimming achievement on day 3 in the Morris water maze test, which was conducted on day 81 post surgery. (**A**) Swimming time, swimming distance, and crossing number in Q1 within 60 s in the probe trial (no platform). Data were analyzed by one-way ANOVA with the multiple-range test. Mean ± SEM. n = 6. $^{**}p < 0.01$, vs. the Sham-operated group. (**B**) The swimming trajectory of rats in the probe trial. (**A**) Sham-operated group, showing greater swimming time and distance in the target quadrant (Q1). (**B**) Composited Aβ-treated group, showing less swimming time and distance in target quadrant (Q1).

Figure 5. Composited Aβ influenced rat swimming speed. The rat swimming speed was calculated by the visible platform trial on day 7 of the Morris water maze test, which was conducted on day 85 after the operation. The rat swimming speed of each group was not significantly different. Data were analyzed by one-way ANOVA with the multiple-range test. Mean ± SEM. n = 6.

Figure 6. Composited Aβ caused rat neuronal morphological change. (**A**) Representative

images of hippocampal and cerebral cortical neurons stained with HE. (**A1–B1**) Hippocampus 40x; (**A2–B2**) Hippocampus CA1 400x; (**A3–B3**) Cerebral cortex 400x. (**A1–A3**) Sham-operated group; (**B1–B3**) Composited Aβ-treated group; shows neuron marked loss, neurofibrillary degeneration (→), neuronophagia (←), nuclear pyknosis (↗), nuclear margination (↙) in hippocampus, typical colliquative necrosis (★), disrupted cellular membranes, large numbers of inflammatory cells infiltrated in the cerebral cortex in part of the composited Aβ-treated group. Scale bar of **A1, B1** = 10 μm; Scale bar of **A2, B2, A3, B3** = 100 μm. (**B**) Numbers of neurons with HE stain in the hippocampus and cerebral cortex, which were counted under a light microscope (400x). Each volume represents mean ± SEM from 9 visual fields of 3 independent samples (n = 3). $^{**}p < 0.01$, vs. Sham-operated group.

Figure 7. Subcellular structure of hippocampal neuron assessed by electron microscopic observation. A1–A4: Sham-operated group. Scale bar of **A1** = 4 μm, 12,000x; Scale bar of **A2** = 3 μm, 15,000x; Scale bar of **A3** = 5 μm, 10,000x; Scale bar of **A4** = 1 μm, 35,000x. (**B1–B4**) Composited Aβ-treated group. (**B1**) Neuron and nuclear pyknosis (⬅), euchromatin condensation or degeneration (#), astrocyte foot swell (*), high electron density mitochondria (▲), myelin sheath layers loose or attenuation (➡); (**B2**) Greater GFAP, high electron density mitochondria (▲), myelin sheath layers loose or attenuation (➡), greater secondary lysosomes (⬆), pericytes pyknosis, pericytes euchromatin condensation or degeneration (☆), astrocyte foot swell (*), high electron density mitochondria (▲), more lipofuscin (⬇), myelin sheath layers loose or attenuation (➡). B4: more excitatory neurotransmitter (##), high electron density or injury membrane mitochondria (▲), less synapsis. Scale bar of **B1, B2** = 10 μm, 5,000x; Scale bar of **B3** = 5 μm, 8,000x; Scale bar of **B4** = 1 μm, 40,000x.

Figure 8. Composited Aβ caused Aβ burden in rat neurons. (**A**) Representative images of positive Aβ neuron stained by Congo red in the hippocampus and cerebral cortical. (**A1–B1**) Hippocampus CA1 400x; (**A2–B2**) Cerebral cortex 400x. (**A1–A2**) Sham-operated group; (**B1–B2**) Composited Aβ-treated group, shows more Aβ positive cells stained by Congo red. Scale bar = 10 μm, 400x. (**B**) Positive numbers of Aβ neurons stained by Congo red in the hippocampus and cerebral cortex, which were counted under a light microscope (400x). Each volume represents mean ± SEM from 9 visual fields of 3 independent samples (n = 3). $^{**}p < 0.01$, vs. Sham-operated group.

Figure 9. Composited Aβ caused NFT aggregation in rat neurons. (**A**) Representative images of positive NFT neurons stained by silver nitrate in hippocampus and cerebral cortex. (**A1–B1**) Hippocampus CA1 400x; (**A2–B2**) Cerebral cortex 400x. (**A1–A2**) Sham-operated group; (**B1–B2**) Composited Aβ-treated group. showing the more NFT positive cell stained by silver nitrate in composited-treated group. Scale bar = 10 μm, 400x. (**B**) Positive NFT neurons numbers of stained by silver nitrate in hippocampus and cerebral cortex, which were counted under a light microscope (400x). Each volume represents mean ± SEM from 9 visual fields of 3 independent samples (n = 3). $^{**}p < 0.01$, vs. Sham-operated group.

DISCUSSION:

It is well known that the loss of learning and memory are major clinical symptoms in AD patients[2]. The procedure described here is an *in vivo* method to study AD; we have adapted a previously published protocol that tested a medication to alleviate memory deficits and neuronal

injuries in a rat model[4]. Our protocol provides important details to obtain valuable data, as well as a high survival rate of animals that successfully model operation, memory deficits, neuron injuries, Aβ burden, and NFT deposition, to mimic AD (in the present experiment, the survival rate and successful model rate of operation are more than 90%). These successful model rats were used to measure their spatial memory with the Morris water maze test. The positioning navigation trial found that the composited Aβ can cause rat memory acquisition impairment; the probe trial found that the composited Aβ can decrease rat memory retention; and the reversal trial found that the composited Aβ can result in rat re-learning impairment. These Morris water maze test data show that the composited Aβ can induce rat spatial memory. Overall, injecting rats intracerebroventricularly with Aβ25-35 in combination with $AlCl_3$ and TGF-β1 created a feasible and credible *in vivo* AD-like animal model for the laboratory.

Previous studies have shown that the brain volume in AD patients is 10% less than that of healthy individuals. Various atrophies can be found in the cerebral hemisphere by visual observation. The degree of cortical atrophy is positively related to the memory impairment[18]. In the histology, the large number of neuron loss and severe morphological pathology directly disturb the memory function in AD patients[19]. In the present study, light/electron microscopic observation found that the rats microinjected with composited Aβ displayed dramatic neuropathological changes, including neuron loss, and neuronal and subcellular structure disruption. This result corroborates the rat spatial memory disorder induced by composited Aβ, and is similar to the state of AD patients.

It is well known that the brain Aβ burden and NFT aggregation are considered the most important histopathogenic traits in AD. They can destroy the neuronal structure, disturb the neural signaling, disrupt the neuronal function, and result in advanced dementia[17]. The present animal model found Aβ burden and NFT aggregation in brain, which agree with the AD patient state. Therefore, the present neuron injuries in rats induced by composited Aβ can be used as a model to study neuronal pathology and treatment strategy of AD.

The following are examples of screening drug effects in AD rat models: Zhao *et al.*, reported that both flavonoids from Scutellaria stems and leaves (SSF) and Scutellaria barbata (SBF) can attenuate rat memory impairment and apoptosis induced by composited Aβ[8,9]. Guo *et al.*, also reported that SBF can inhibit NFT aggregation and tau protein over-phosphorylation at Ser199, Ser214, Ser202, Ser404, and Thr231 side, and decrease GSK-3β, CDK5, and PKA protein and mRNA expression in composited Aβ-treated rats[20]. Simultaneously, Shang *et al.*, have also reported that SBF can suppress the astrocyte and microglia proliferation, and lower Aβ1-40, Aβ1-42, and β-site APP cleaving enzyme 1 (BACE1) mRNA expression in the brain of composited Aβ rats[21]. Based on the above results, our animal model is advantageous over other AD-like model, which involve more neuronal function and structure disorder.

Concerning other AD-like model, single intracerebroventricular injection of Aβ to rats can cause rat memory deficits, neuron loss, and neurogliocyte proliferation, but may or may not have Aβ and NFT deposition[22]. Rats exposed to high dose Al appear to have a high success rate, mimic AD, and a cost-effective animal model, with memory impairment, neuron loss, neurogliocyte proliferation, and senile plaque (SP) and NFT aggregation in the brain. However, the high dose of Al may cause rat liver injuries and anorexia, accompanied with decreased weight[23]. The aged

rat is another AD-like model. The aged rats demonstrate memory deficits, neuronal structure/substructure pathological changes, lipofuscin deposition, but without Aβ burden and NFT aggregation. Rats of more than 24 months of age are considered aged for this model, and therefore it requires a longer period of feeding and thus the cost is higher[17,24]. SAMP8 and APP transgenic mice are the closest mimic to AD and they are the most ideal models for investigating AD. But both animal models are higher priced and are limited to use in the laboratory[25,26]. Compared with the above animal models, our model of composited Aβ-treated animal model has a lower cost and high performance, making it an ideal tool for studying AD.

In conclusion, intracerebroventricular injection of Aβ25-35 combined with $AlCl_3$ and TGF-β1 to rats offers a valuable *in vivo* animal model to better understand the spatial memory impairment, neuronal injuries, Aβ burden, and NFT deposition underlying AD. This model provides a fast and relatively simple experimental protocol with a high animal survive rate and high model successful rate of operation, as well as a high rate of duplication, which showed to be more economic. The present animal model is an effective model to mimic AD and can further validate itself by being used to mimic various other diseases.

DISCLOSURES:
The authors have nothing to disclose.

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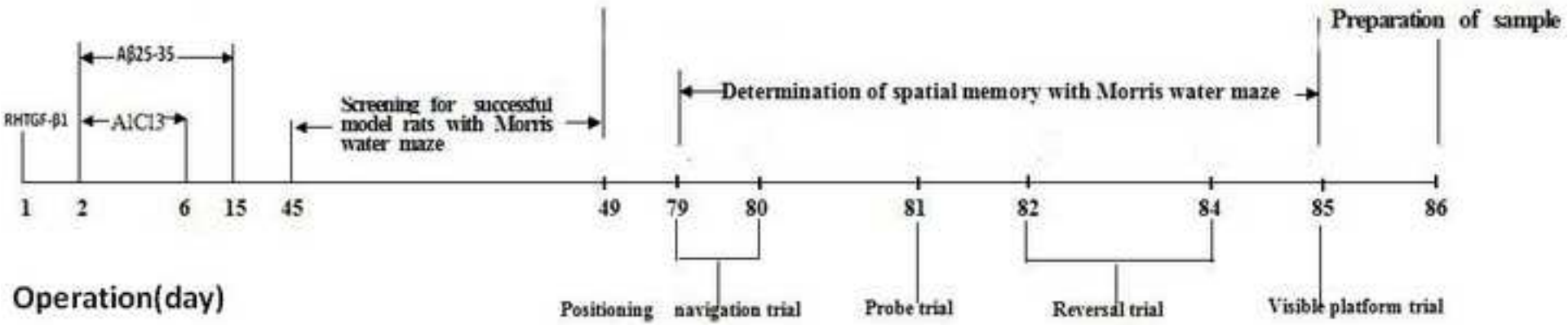

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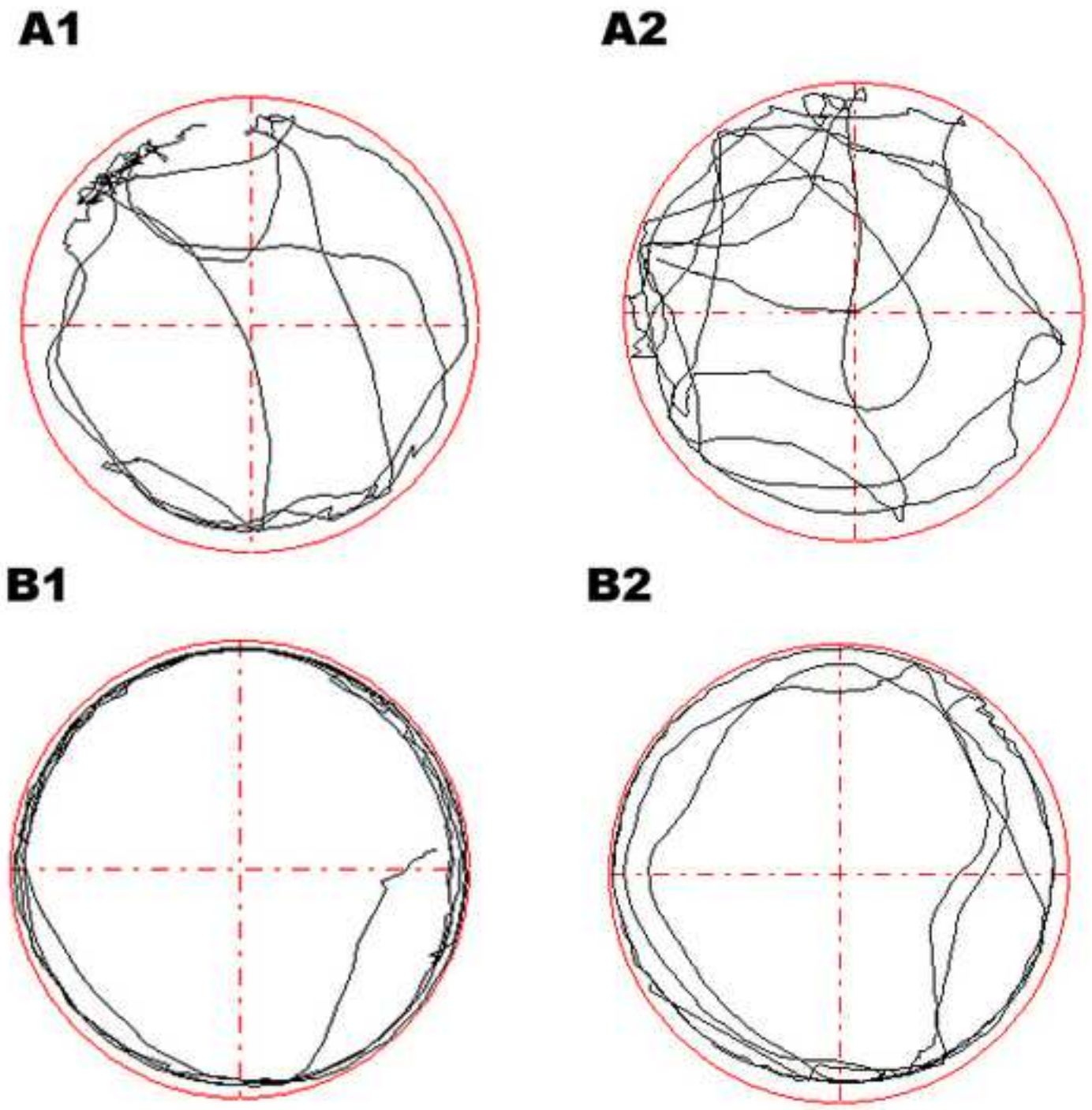

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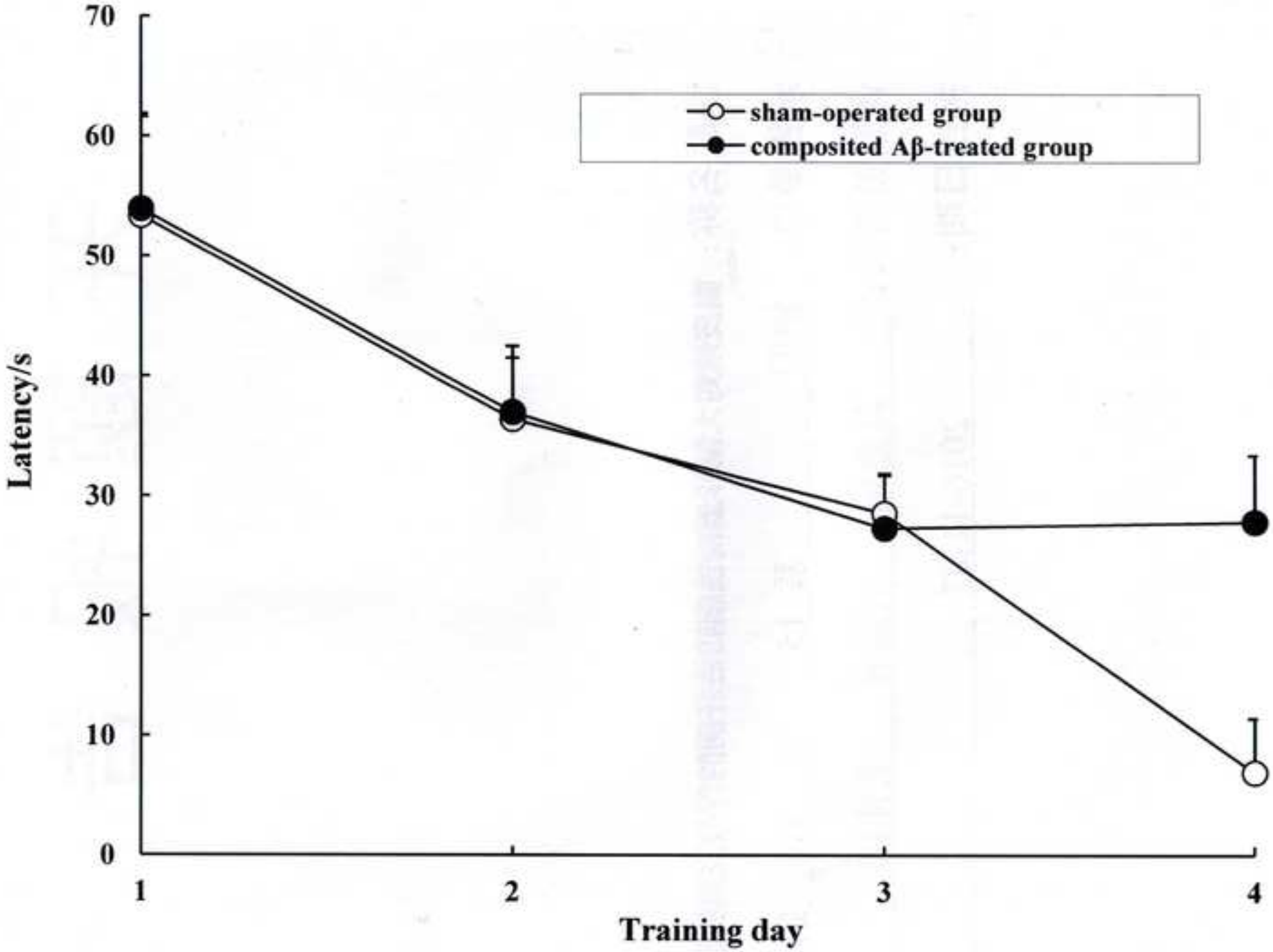

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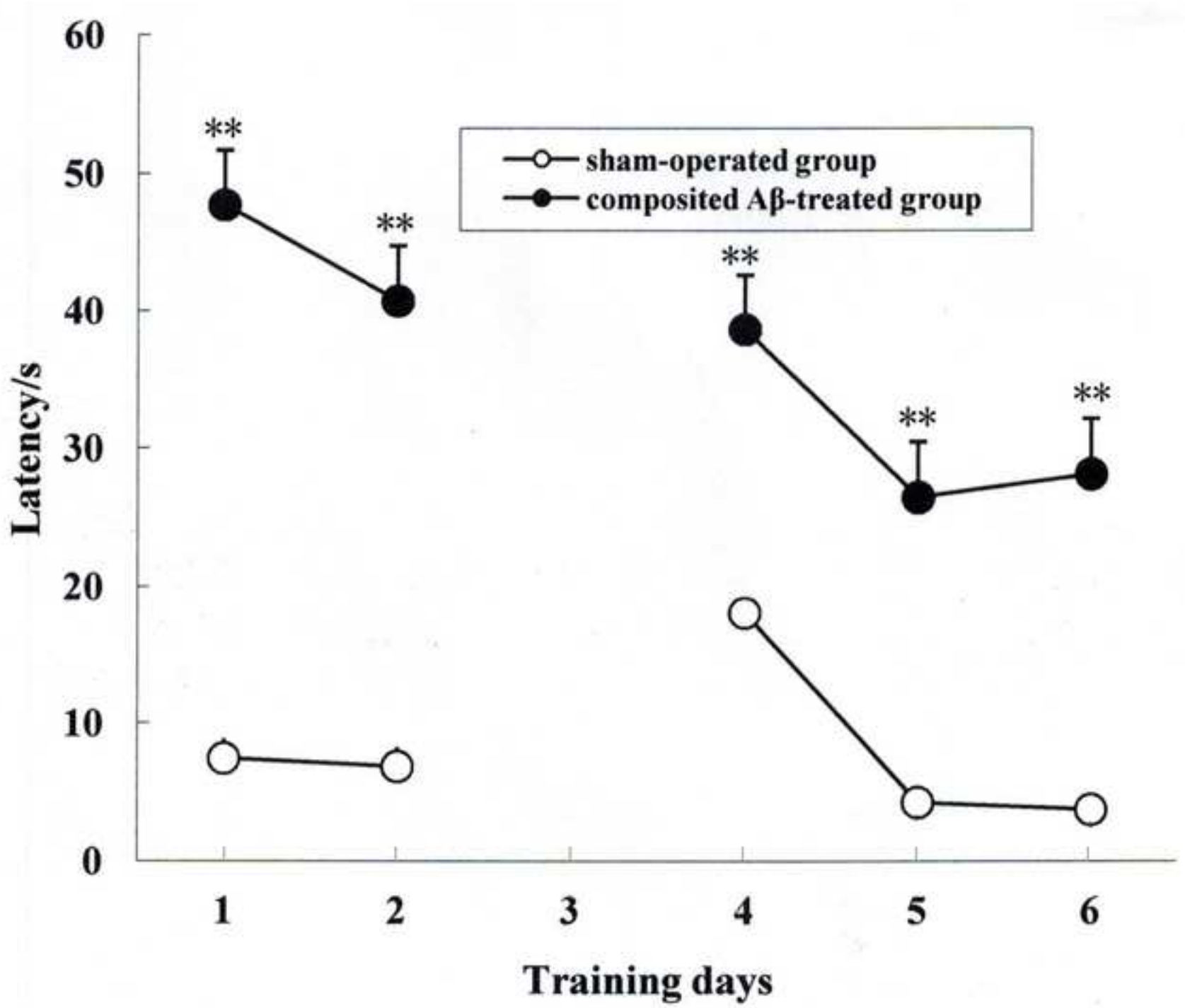

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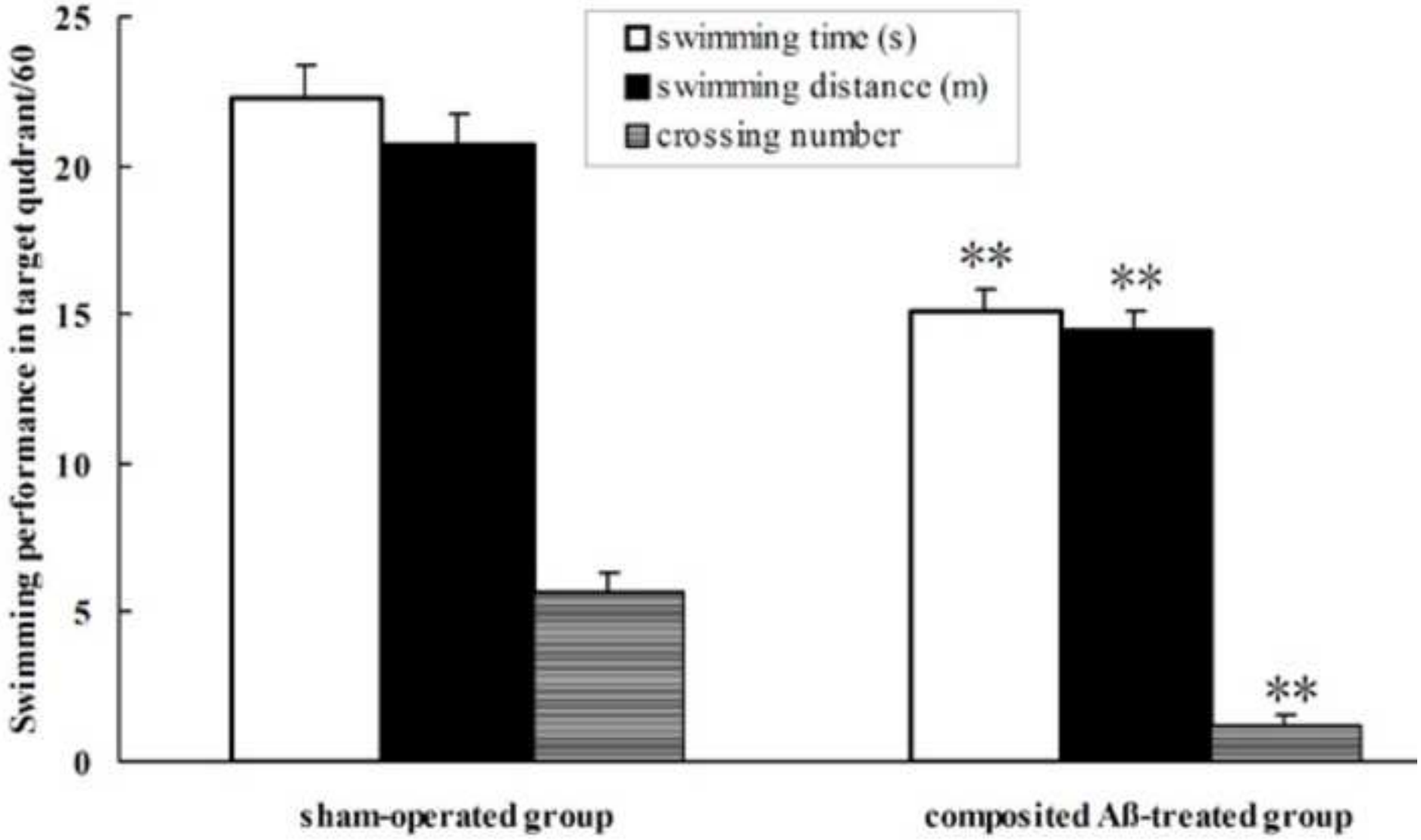

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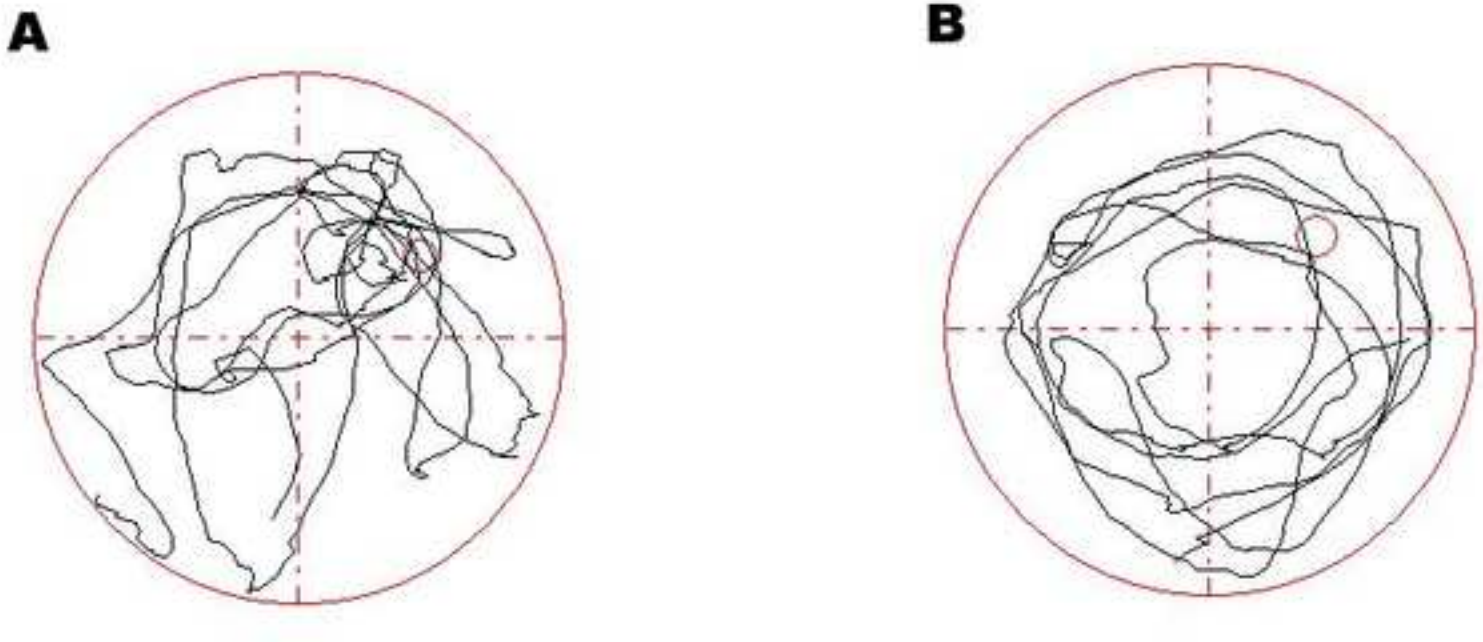

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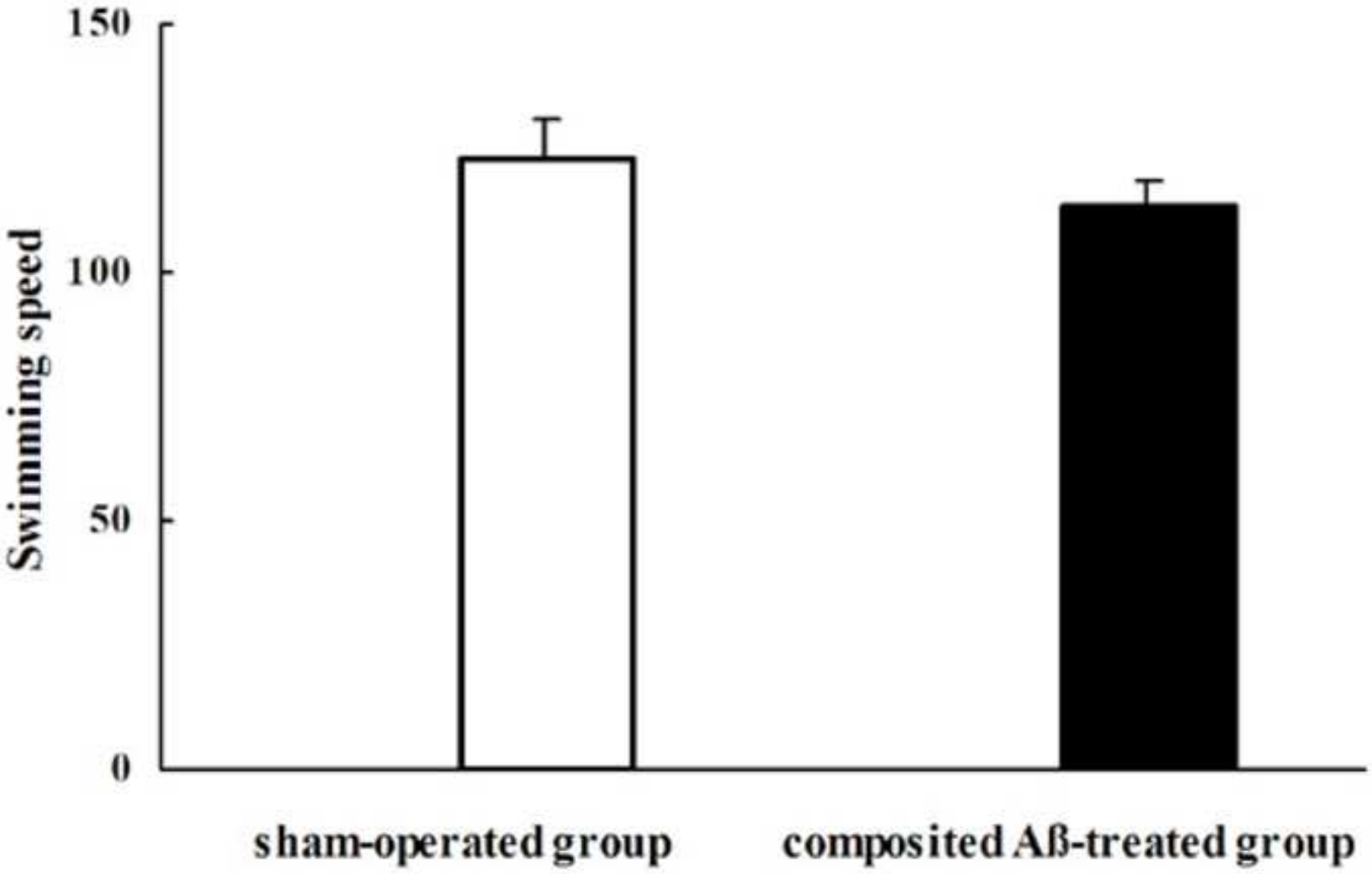

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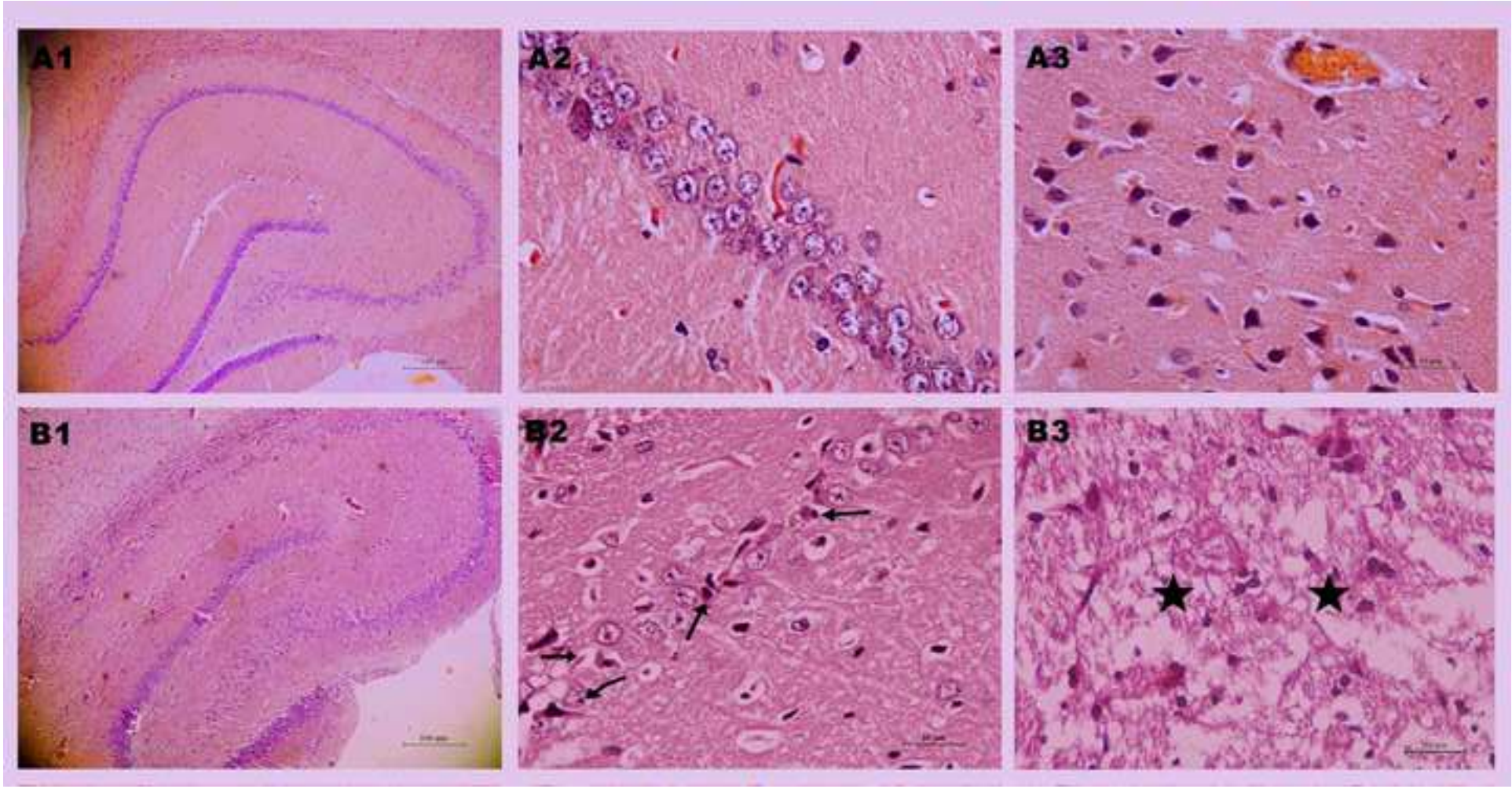

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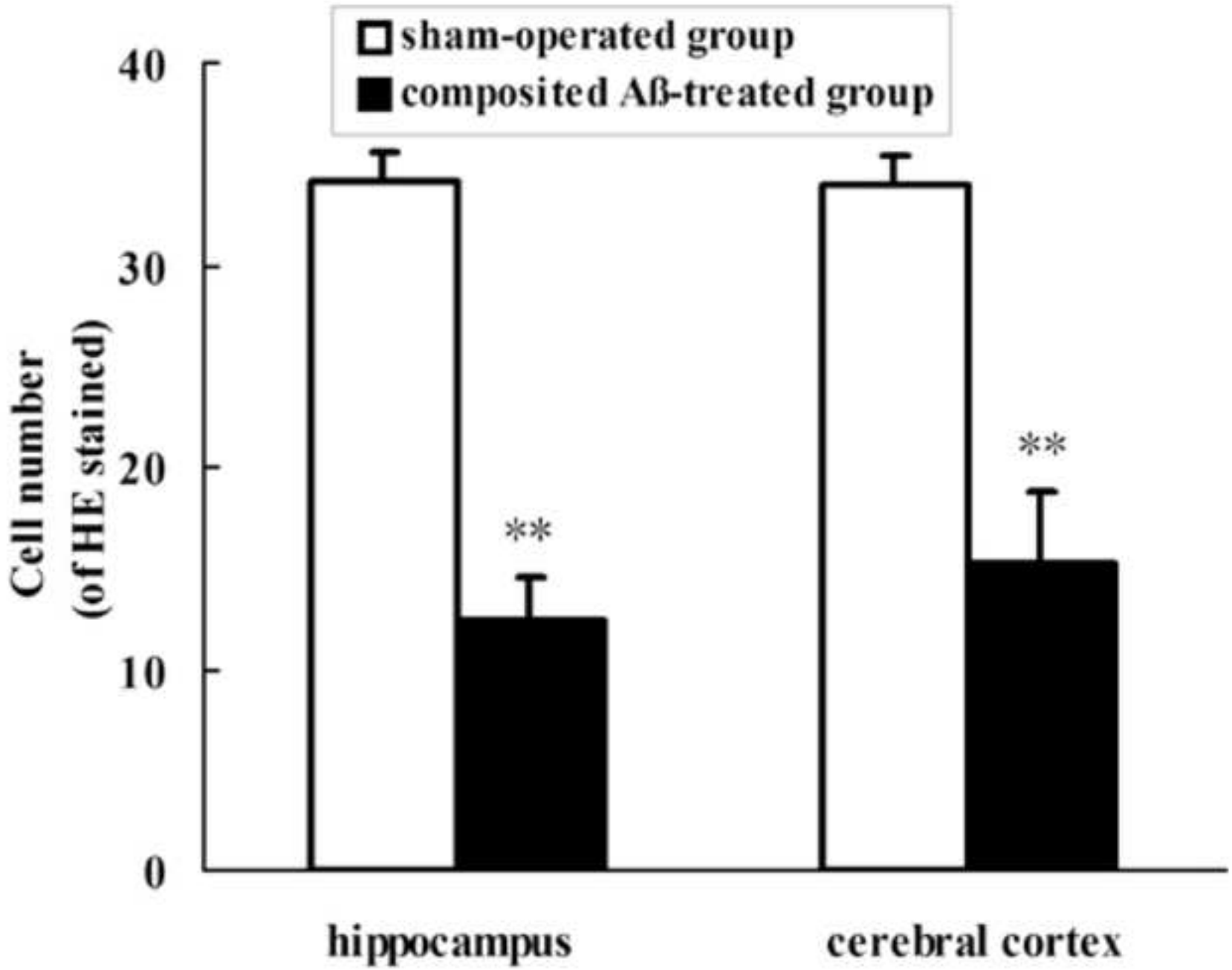

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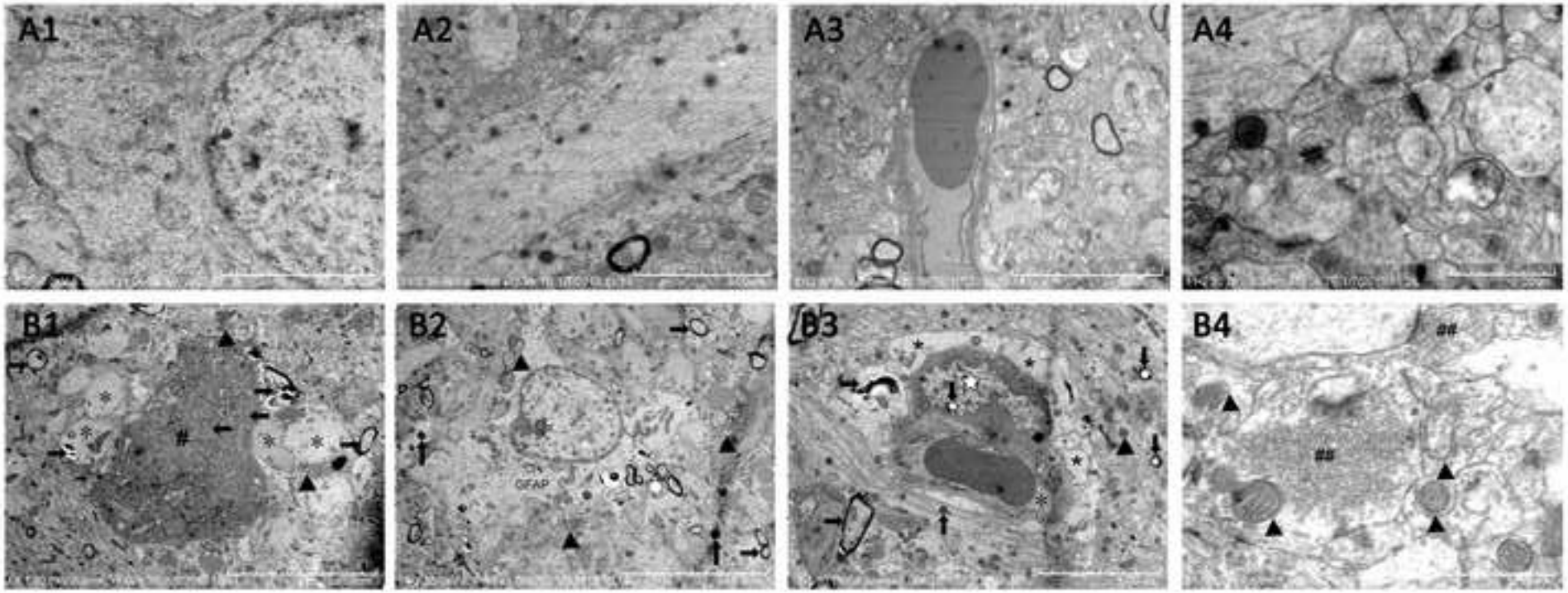

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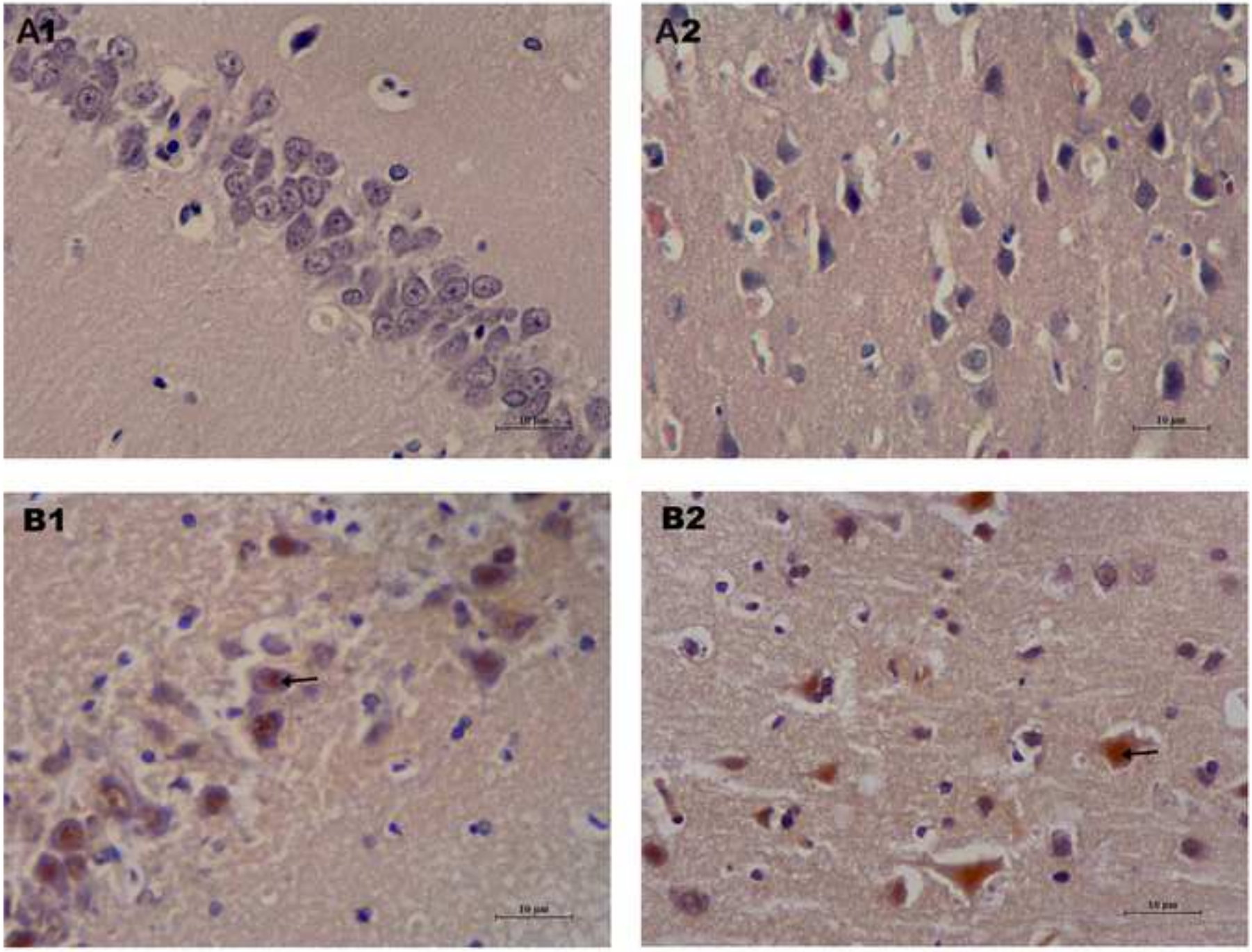

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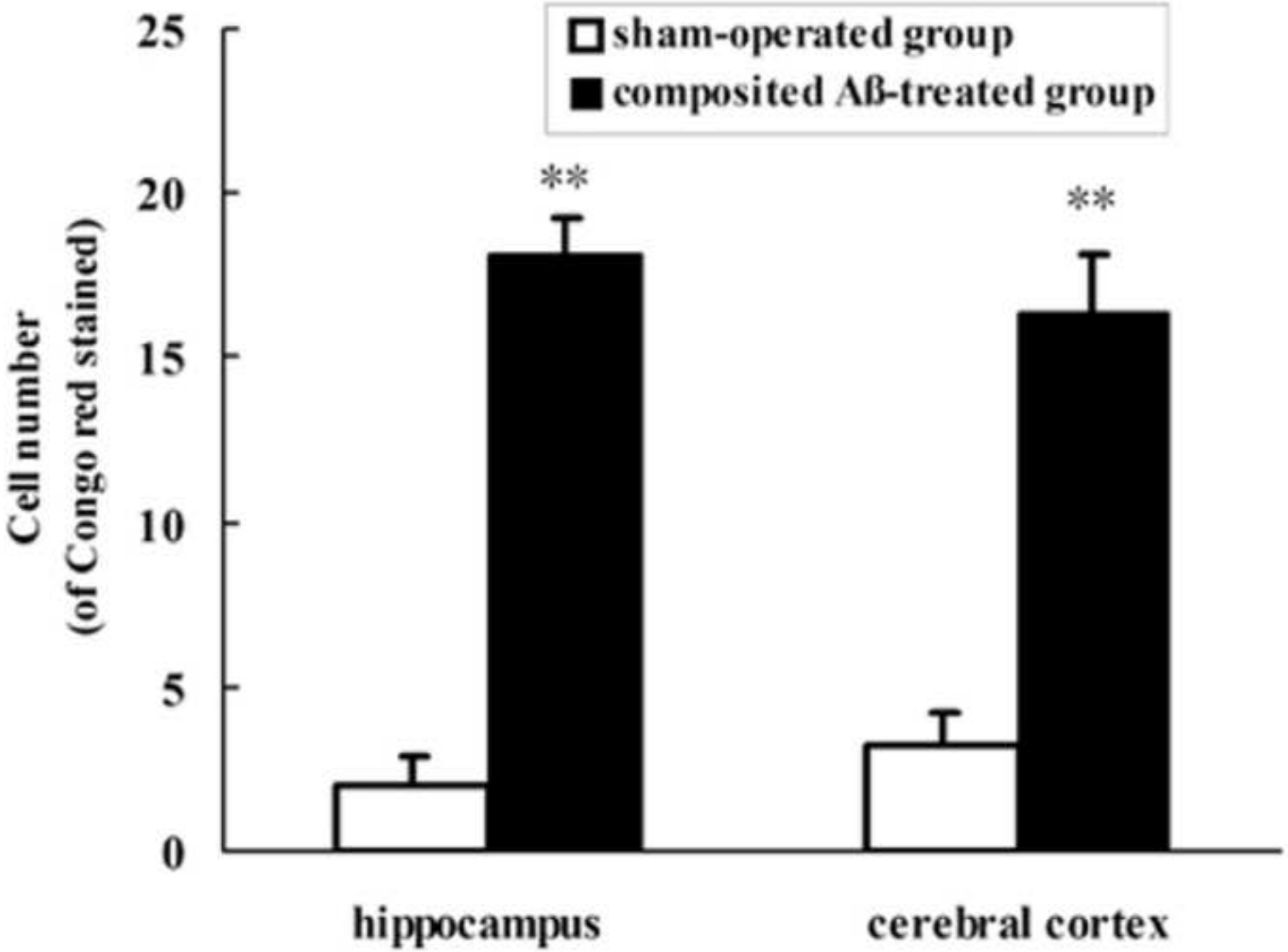

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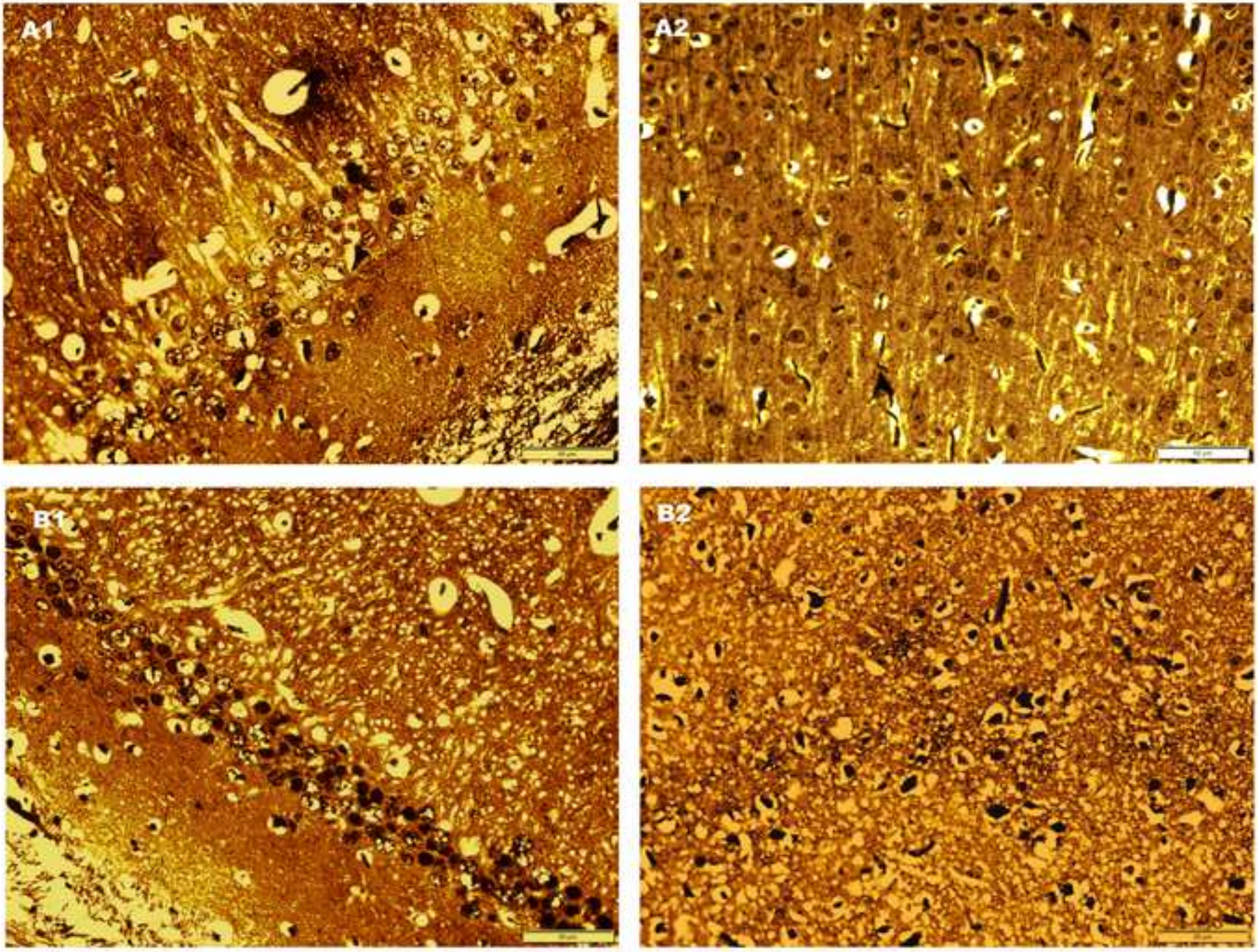

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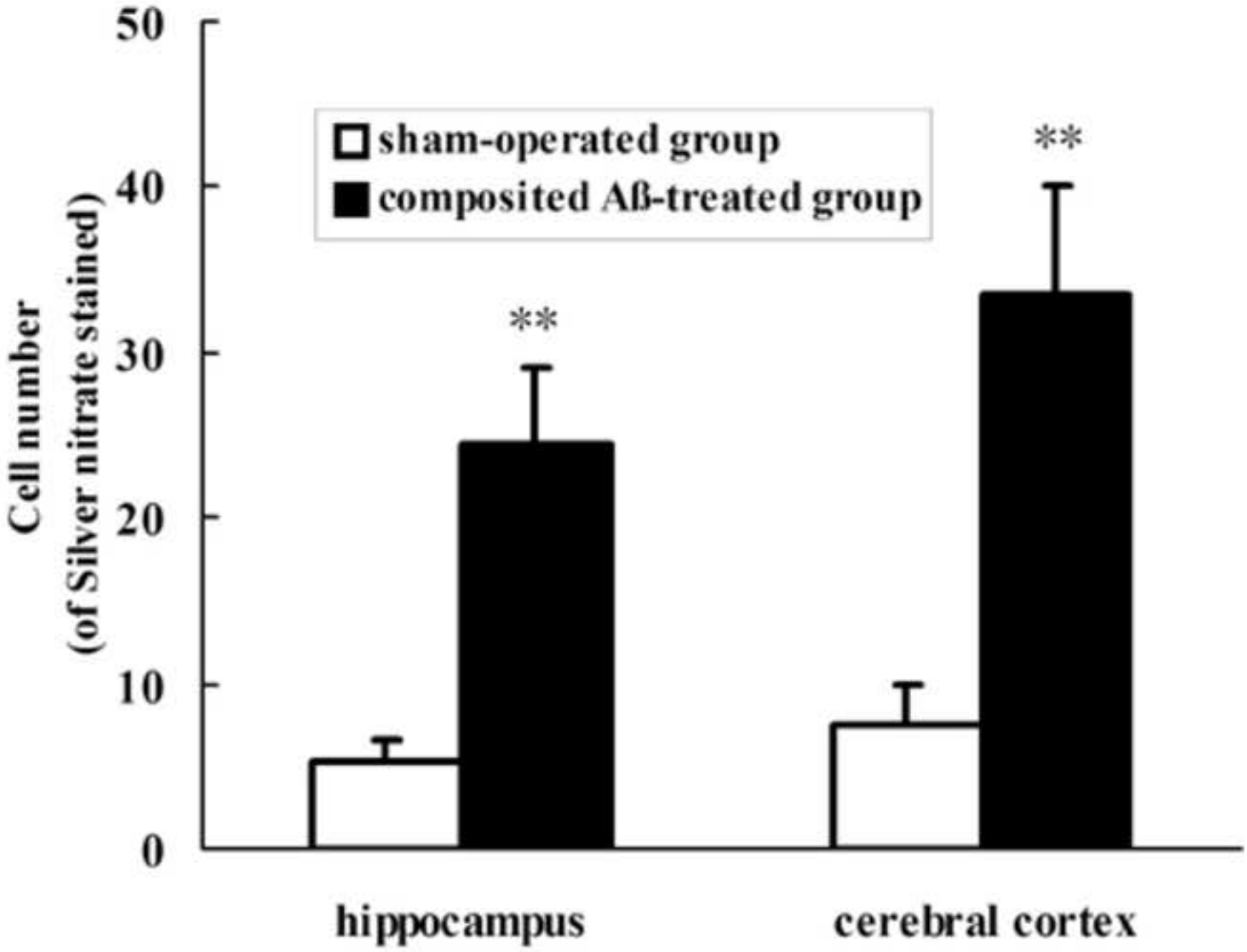

Name of Material/ Equipment	Company
Sprague-Dawley rat	Beijing Vital River Laboratory Animal Technology C
Morris water maze	Chinese Academy of Medical Sciences and Peking Union Medical Research Institute, China
Movable small animal anesthesi	RWD Life Science Co., Ltd. China
Brain Stereotaxic Apparatus	RWD Life Science Co., Ltd. China
Flexible bone drill	Shanghai Soft Long Technology Development Co., Ltd. China
Transmission electron microscope	Japan Co., Ltd. Japan
Two channel microinjection pump	RWD Life Science Co., Ltd. China
EM microtome	Hitachi Co., Ltd. China
Dummy cannula	RWD Life Science Co., Ltd. China
Guide cannula	RWD Life Science Co., Ltd. China
Internal cannula	RWD Life Science Co., Ltd. China
Tighten the nut	RWD Life Science Co., Ltd. China
Fixing screw	RWD Life Science Co., Ltd. China
The screwdriver	RWD Life Science Co., Ltd. China
PE Tubing	RWD Life Science Co., Ltd. China
Amyloid beta 25-35	Sigma Aldrich Co. USA
Recombinant human transforming growth fa	PeproTech Inc. USA
Aluminium trichloride	Tianjin Kemiou Chemical Reagent Co., Ltd. China
Congo red	Tianjin Kemiou Chemical Reagent Co., Ltd. China

Silver nitrate	Sinopharm Chcmical Reagent Co., Ltd. China
Zinc phosphate dental cement	Dental Material of Factory Shanghai Medical Instruments Co., Ltd. China

Catalog Number	Comments/Description
SCXK(Jing）2012-0001	300–350 g
No	
R580	
68001	
BW-sD908	
JEM-1400	
RWD202	
H-7650	
62001	0.D.0.64×I.D.0.0.45mm/M3.5
62101	0.D.0.40mm/M3.5
62201	0.D.0.41×I.D.0.25mm/配合M3.5
62501	0.D.5.5mm/L7.5mm/M3.5
62514	M1.2×L2.0mm(100BAO)
62999	45*1mm
62302	
SCP0002-5MG	
100-21	
3011080	
3010016	

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Article Title: Establishment of an ideal simulated Alzheimer's disease animal model by intracerebroventricular injection of composited Aβ

Signature: Shang Yazhen Date: 28/Feb. 2017

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JoVE Editor

The cover letter describing on manuscript:

Establishment of an ideal (valuable) mimic Alzheimer's disease animal model by intracerebroventricular injection of composited amyloid beta protein" (Wu Xiaoguang, Cheng Jianjun, Wang Xiaoqing, Gao Yang, Shang Yazhen. JoVE 56157) in detail which editor and reviewers concerned. The correction in the revision was blue words and highlight steps were in yellow background.

The manuscript has been modified by the Scientific Review Editor to comply with the JoVE formatting standard. Please maintain the current formatting throughout the manuscript. The updated manuscript (56157_R0_03082017) is in your Editorial Manager account. In the revised PDF submission, there is a hyperlink for downloading the .docx file. Please download the .docx file and use this updated version for any future revisions. The updated manuscript is also attached.
Reply: Authors corrected this in the revision according to editors' suggestion.

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Reply: Authors corrected this in the revision according to editors' suggestion.

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Reply: Authors provided other new figures and data in revision and not ask the previous publisher.

3) All commercial products should be sufficiently referenced in the table of

materials/reagents. Please provide company, catalog number and other details in Table of Materials for – medical bone wax, drill, xylene, alcohol, hematoxylin, eosin, glutaraldehyde, PBS, osmic acid, methylene blue,
Reply: Authors corrected this in the revision according to editors' suggestion.

4) Please copy-edit the entire manuscript for any grammatical errors you may find. The text should be in American-English only. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol and the manuscript. Please thoroughly review the language and grammar prior to resubmission. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.
Reply: Authors ask a native English speaker to correct the manuscript.

5) Please provide e-mail addresses of all authors on the first page
Reply: Authors corrected this in the revision according to editors' suggestion.

6) Please spell out all abbreviations at their first appearance – PE pipe,
Reply: Authors corrected this in the revision according to editors' suggestion.

7) Please format your manuscript as per JoVE's author template (attached)
Reply: Authors corrected this in the revision according to editors' suggestion.

8) Kindly refer to a representative manuscript that was published in JoVE [attached] and write your manuscript as per this style.
Reply: Authors corrected this in the revision according to editors' suggestion.

9) Do not use abbreviations in the Title – spell out Aß
Reply: Authors corrected this in the revision according to editors' suggestion.

10) There is a significant overlap in text to your previously published work. When you seek help of copy editing services, please minimize this overlap.
Reply: Authors corrected this in the revision according to editors' suggestion.

Abstracts –
1) Line 26 - Please add a Short Abstract (10-50 words) that clearly states the goal of the protocol.
Reply: Authors corrected this in the revision according to editors' suggestion.

2) Line 26 - Make sure that Short Abstract clearly states the goal of the protocol. For example, "This protocol/manuscript describes…"
Reply: Authors corrected this in the revision according to editors' suggestion.

3) Line 29 - The abstract in the current version should be re-labeled as long abstract
Reply: Authors corrected this in the revision according to editors' suggestion.

4) Line 30 – spell out AD
Reply: Authors corrected this in the revision according to editors' suggestion.

5) Line 37 – 25-35 …. what is this? Molecular wt? amino acid residues?
Reply: ----25-35 is amino acid residues.

6) Line 47 – please delete this link – it does not link to your video
Reply: Authors corrected this in the revision according to editors' suggestion.

• Introduction:
1) Line 54 – reference formatting - Please make sure that your references comply with JoVE instructions for authors. In-text formatting: corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text of the manuscript. Citation formatting should appear as follows: (For 6 authors or less list all authors. For more than 6 authors, list only the first author then *et al.*): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source*. **Volume** (Issue), FirstPage – LastPage, doi:DOI (YEAR).]
Reply: Authors corrected this in the revision according to editors' suggestion.

2) Line 54 – spell out Aß.
Reply: Authors corrected this in the revision according to editors' suggestion.

3) Please expand your Introduction to include the following: 1) The rationale behind the development and/or use of this technique 2) The advantages over alternative techniques with applicable references to previous studies 3) Description of the context of the technique in the wider body of literature and 4) Information that can help readers to determine if the method is appropriate for their application.
Reply: Authors supplemented the related the information in revision.

• Protocol:
1) Line 80 – label as note
Reply: Authors label the line 80 as note in the revision.

2) Line 95 – 99 – label as note
Reply: Authors label the line 99 as note in the revision.

3) Line 100 – this should be 2.1 - Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 2. should be followed by 2.1. and then 2.1.1. if necessary and all steps should be lined up at the left margin

with no indentations. There must also be a one-line space between each protocol step.
Reply: Authors adjust the numbering of protocol section in the revison.

4) Line 101 – intraperitoneal injection
Reply: Authors corrected this in the revision.

5) Line 104 – make an incision
Reply: Authors corrected this in the revision.

6) Line 109 – what do you use to drill a hole?
Reply: Authors supplemented the related information this in the revision.

7) Line 124 – guide
Reply: Authors corrected this in the revision.

8) Line 129 - Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.
Reply: Authors corrected the unit and added a space between the numerical value and unit in the revision.

9) Line 135 – what are the anesthesia conditions?
Reply: Authors supplemented the anesthesia condition. (…under inhalation anesthesia with isoflurane every day)

10) Line 141 – do you administer anesthesia every day while injecting Aß / AlCl3?
Reply: Yes.

11) Line 147 – please specify the type of suture
Reply: Supplement the type of suture in the revision.

12) Line 152 – specify the diet
Reply: The routine diet for rats.

13) Line 160 – label as note
Reply: Authors label the line 160 as note in the revision.

14) Line 156 onwards – Please re-write steps of your protocol section in imperative tense, as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.). For example, write line 163 as, “blacken the pool water with several drops of ink.” Line 162 – “Maintain the depth of water at 31.5

cms and the temperature a 23± 1 dec C”
Reply: Authors corrected this in the revision according to editors’ suggestion.

15) Line 199 – this should be 3.3.1
Reply: Authors corrected this in the revision according to editors’ suggestion.

16) Line 233 – replace “killed” by “sacrifice”
Reply: Authors corrected this in the revision according to editors’ suggestion.

17) Line 240 – please add “caution: xylene is toxic. Perform the following steps in a fume hood”
Reply: Authors corrected this in the revision according to editors’ suggestion.

18) Line 241 – 246 – please provide details such as concentration of dyes, time of incubation for dewax, etc
Reply: Authors supplemented the related information.

• Representative Results:
1) Line 270 - What are the A1, A2, B1, B2 panels in figure 2A? please explain
Reply: A1, A2, B1, B2 panels in figure 2A are the adaptive swimming trajectory of rats in the Morris water maze and supplemented in the revision. The explain is in legend.

2) Line 297 – in figure 4B – please define which panel represents the sham-operated and composited Aß group.
Reply: Authors define the sham-operated (4BA) and composited Aß group (4BB).

3) Move lines 303 -309 to line 288 as it pertains to data shown in Figure 3
Reply: Authors corrected this in the revision according to editors’ suggestion.

4) Line 338 – do you mean figure 5B?
Reply: Yes, Figure 5B is correct.

• Figure legends:
1) Move figure legends to line 356
Reply: Authors corrected this in the revision according to editors’ suggestion.

2) Line 463 – are all the figures re-used? If not, then move this line to the specific figure legend that is re-used.
Reply: Authors corrected this in the revision according to editors’ suggestion.

• Discussion:
1) Please expand the discussion so that it covers the following in detail and in

paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, and 3) critical steps within the protocol.
Reply: Authors added the related information in discussion in revision.

• Figures:
1) Figure 5A – make the scale bar thick and specify the scale bar length with the appropriate panel. For example, scale bar A1B1 – 10 um, A2-B3 – 100 um.
Reply: Authors corrected this in the revision according to editors' suggestion.

2) Figure 6B – make the scale bar thick, they are not visible. What does the open / unfilled star mark in figure 6B?
Reply: Authors corrected this in the revision according to editors' suggestion.

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Reply: Authors corrected this in the revision according to editors' suggestion.

• Highlighting:
1) After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. Please highlight 2.75 pages or less of text (which includes headings and spaces) in yellow background, to identify which steps should be filmed to tell the most cohesive story of your protocol steps. Please see JoVE's instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.
Reply: Authors corrected this in the revision according to editors' suggestion.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
Xiaoguang et al. described in the present article an alternative approach to model AD in rats. As a first experimental step, they injected RHTGF-β1 in order to enhance secondary Aβ aggregation. Then, they co-injected Aβ25-35 peptides (4μg) and AlCl3 (3μL, 1%) through intracerebroventricular way. Finally, they characterized cognitive and histological consequences 2 months after Aβ25-35

injection end.

Major Concerns:

-I suggest to the Authors to avoid some "subjective" terms, thus they used "ideal" in the title and in the abstract. This strong word appears not justified and not appropriate regarding the results obtained.

Reply: Authors altered “ideal” into “valuable” in the revision according to reviewer’s suggestion.

-In the present article, the authors never compare their alternative model with classical ones (i.e. transgenic rats or single Aβ25-35 injection). I suggest they discuss the main advantages of their modeling approach in comparison to already described ones and also the specific drawbacks (for example, the suggested approach appears less easy to use than current AD rat models).

Reply: Authors supplement the related information in the discussion in revision.

-If memory deficit appeared in more than 90% of AD rats, why they add a screening step for successful rat model? On another side, they injected 20 rats with Aβ25-35, and found a success rate of 94.5%. Could authors explain this percentage, if 19 rats on 20 were considered as successful rat model, the rate should be 95% isn't it?

Reply: The aim of this present protocol is to provide a new method for simulated AD model in rats. For examine the rats’ memory acquisition, memory retention and memory re-learning, **we must be sure** the rats injected composited Aβ were model successful rats, then we add the screening step for successful rat model began on the day 45 after operation.

20 rats were subject to the injection of composited Aβ and 19 rats survived and the successful rate of operation is 95%. 19 rats were brought into the model screening and 18 rats were successful model rats. Then, the model successful rate was 94.7%.

-It is missing some control groups such as rats only injected with Aβ25-35,as well rats injected with Aβ25-35 peptides and AlCl3, in order to argue the added value of this modeling approach.

-The authors never justified how this AD rat model could be considered as "most close to the stages of clinical AD patients" than other modeling approaches?

Reply: Yes, we did not design the groups of injected with Aβ25-35, Aβ25-35 peptides and AlCl3 for comparison to ours. Then, we supplement the related information in the discussion.

Minor Concerns:

-With a cell death rate of more than 60%, I wonder how authors imagine the effect of tested drugs in this model? Which kind of therapeutic strategies could be tested in their model (cellular graft, BACE1 inhibitors, amyloid aggregation

disruptors, Tau kinases inhibitors, growth factors and others)? They must discuss this specific aspect.
Reply: Authors added the related results in discussion in the revision.
N/A

Reviewer #2:
Manuscript Summary:
I have reviewed the paper of Wu and colleagues entitled "Establishment of an ideal simulated Alzheimer's disease animal model by intracerebroventricular injection of composited Aß".
This paper describes the rationale and validation of a new toxic rodent model of

Additional Comments to Authors:
Alzheimer's disease (AD) based on the co-injection of Aß + aluminium in the ventricles associated with TGFß1 in the thalamus. The authors provided a detailed procedure for implementing the model, mainly based on brain surgery procedures (stereotactic microinjections). The authors also presented a functional and morphological validation of their model (behavioral assessment in a Morris watermaze- MWM paradigm; neuropathological examination).

Major Concerns:
While potentially of interest, the present report suffers important drawbacks that preclude its publication.

1- The overall interest of the model is overestimated. The use and previous description of this model is very limited (only few papers in the literature). I do not believe that this model mimics AD physiopathology. The induced pathology has only vague resemblance with the standard AD brain lesions (no evidence of amyloid pathology, no tau positive lesions). It appears more reasonable that the toxic treatments induced some kind of severe encephalopathy with both detrimental effects on behavior and on brain morphology. This is not sufficient to conclude that "the spatial memory functional and pathological changes in this model rats are the most close to the states of clinical AD patients".
Furthermore, there are some imprecisions / lacking information about the model's construction. Figure 1 describes the timeline of brain injections with a mention of Aß and aluminium salts but what about TGF-1ß?... In this figure, what is the upper timeline?
Reply: Author cited the more report upon the model application and provided another timeline including TGF- ß1 injection in vision.

No precise information is given about the state/conformation/way of preparation of infused Aß. These are critical missing information.
Reply: Author supplements the information of Aß25-35 in revision.

In Figure 5 I am not convinced at all by the presence of "neurofibrillary degeneration" in brain tissue of treated rats. The authors see this with the eyes of faith but should really provide better demonstration.
Reply: Authors added the results in Aß and tau protein lesion in the model and revised the inappropriate wording, and supplemented infused Aß information in the revision.

2- The manuscript should be completely and carefully edited by a native English speaker. Grammatical and syntactic errors are so numerous that they cannot be listed here or tentatively corrected by reviewers. It reaches such a point that some sentences are even not understandable leaving number of gaps in the description and discussion of the experimental data.
Reply: Ok, authors asked a native English speaker to help revise the manuscript grammatical and syntactic errors.

Minor Concerns:
1-The notion of "simulated model" is debatable. By essence an interventionist model is… simulated.
Reply: Mimic model was instead of simulated model in revision.

2-Reference to an exotic brain atlas for stereotactic surgery (Bao, X.M & Shu,S.Y. The stereotaxic atlas of the rat brain, People's medical publishing house. 1991) is of limited interest for the world-wide research community. International standard remains Paxinos-Watson atlas.
Reply: We refer the brain atlas for stereotactic surgery (Bao, X.M & Shu,S.Y. The stereotaxic atlas of the rat brain, People's medical publishing house. 1991) and also provided the coronal section chart as attachment. So, we can not provide the Paxinos-Watson atlas as the reference.

3-The use of a small pool (120 cm) for rats has to be justified. Same for ink (!) to opacify water. Same for very short inter-trials intervals (10s!). These are not standards procedures.
Reply: The Morris water maze was purchase from Institute of Materia Medica, Chinese Academy of Medical Sciences and the operating instruction was as the above. We used this apparatus to publish lots of paper. Authors cann't provide other watr maze apparatus and operating procedures.

4-what is the "computer-based graphics analytic software" used for videotracking in the watermaze?
Reply: The software was installed in a computer and which linked a video camera upon the water maze. Then the rats swimming behavior in the water maze was recorded.

5-MWM protocol has to be described in the § "Screening for successful memory

impairment of composited Aβ-treated rats", not one page later.
Reply: MWM protocol has been moved to the "model screening" part in revision.

6-When using animals, performing ultrastructural analysis (electron microscopy) without fixation by intracardiac perfusion is questionable. Quality is really reduced with samples fixed by immersion. For instance it is claimed that in treated rats "myelin sheath layers were loose or attenuated" but myelin sheats are not visible in the control condition, precluding any comparisons…
Reply: Authors provided more pictures (electron microscopy) to show more overall information in revision.

7-line 274 : fig 2B represent response latencies in the MWM, not "percentage of successful model rats" as stated in the text.
Reply: Authors supplemented the related information in the revision.

8-line 311: why claiming "composited Aß influenced the rats' swimming speed" while demonstrating the opposite? By the way, showing same latencies in the different groups does not mean that animals display the same speed (speed = distance / time). The speed (or distance) measures should be analyzed and presented.
Reply: Ok, Authors correct the related information according to reviewer' suggestion.

9-Figure 5A: even if already published in the original plagiarized paper, microphotographs are of bad quality with uneven illumination indicating that the condenser and diaphragm were not correctly adjusted (I would recommend to follow Köhler illumination procedure before taking photos).
Reply: Authors have tried best to provide the best microphotographs in revision.

Additional Comments to Authors:
N/A

Reviewer #3:
Manuscript Summary:
The authors described in detail an already published protocol to produce and characterise a rat model for Alzheimer's disease (AD) based in the intracerebroventricular injection of the amyloid beta protein 25-35 (Aß25-35). Combined injection of Aß and aluminum trichloride (AlCl3) into the lateral ventricle and recombinant human transforming growth factor-ß1 (RHTGF-ß1) into the anterodorsal thalamic nucleus favors Aß deposition in the hippocampus, simulating pathological features as well as memory deficits occurring in AD.
Methodological differences in Aß infusion are a great source of inconsistency and lack of reproducibility that make it difficult to clearly assess the effect of a specific drug or treatment. In this context, a precise protocol describing not only

the infusion, but also the pathological and behavioural readout might be of interest in the field for testing in a reproducible way new disease-modifying compounds.
The details of the procedure are well described and the timeline of the experimental design clear and a very helpful as overview of the process. However, there are number of issues that should be addressed:

Major Concerns:
1. The experiment is controlled by saline-injected rats. However, it is well described that the vehicle in which Aß is diluted might have a toxic effect itself. Thus, the proper control for these kind of experiments should be rats injected with the vehicle or with scramble peptides. The vehicle used should be also mentioned in the protocol.
Reply: Authors add the preparation (dissolution method) of Aß25-35, AlCl3 and TGF-ß1 for explaining the controlled rats by saline-injected, but vehicle or with scramble peptides.

2. The discussion is somehow poor. Limitations of the procedure (such as Aß maximum volumes that can be injected and diffusion capacity), together with advantages of this infusion method compared to alternative approaches should be addressed in this section. And the same for possible variations in the protocol, for example, has it being tested what happens if already aged animals are infused following this protocol?
Reply: Authors add the related information in discussion in revision. In the future, we may try to use the protocol to already aged animals and observe what happen?

3. Errors in English grammar make it hard to read some parts of the manuscript.
Reply: Authors asked a native English speaker to help revise the manuscript grammatical and syntactic errors.

Minor Concerns:
1. Critical steps should be highlighted and some troubleshooting for common difficulties provided along with the protocol.
Reply: Yes, Critical steps have be highlighted with yellow background. The troubleshooting has been provided in the discussion in revision.

2. A description of recovery measures and motorisation of animals after surgery would be useful.
Reply: Authors add the related information in revision.

3. Figure 4B has panels A and B.
Reply: Yes, Figure 4 panels A and B.

Additional Comments to Authors: N/A

JoVE Editor

The cover letter describing on manuscript:

“Establishment of a valuable mimic Alzheimer’s disease animal model by intracerebroventricular injection of composited amyloid beta protein” (Wu Xiaoguang, Cheng Jianjun, Wang Xiaoqing, Gao Yang, Shang Yazhen. JoVE 56157) in detail which editor concerned. The correction in the R1- revision was red words and highlight steps were in yellow background.

Editorial comments:

Changes recommended by Review Editor JoVE 56157_R1:
Thank you very much for the revisions that were made to the manuscript in response to the Editorial comments. We appreciate your patience in working with us to ensure that the manuscript complies with JoVE’s unique format and requirements. While majority of the Editorial comments have been addressed in your previous revision, the following comments need to be addressed in order for your manuscript to proceed to the next step of the review process. In the editorial manager, there is a hyperlink to download the .docx file. **Please download the .docx file and use this updated version for future revisions.**

1) Since you are re-using figures from previous publications, please obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section.
Reply: I asked the previous publisher and got their reply for re-use Figure, and will be provided a Word document as a Supplement file along with re-submission.

2) Editor modified the formatting of the manuscript and adjusted spacing of the protocol section. Please read the entire manuscript carefully and make changes if required. Do not change the formatting.
Reply: Authors comply with editor suggestion.

3) Editor added track changes to the word document of your manuscript, attached to this email. Please approve/revise all track changes; however, do not accept the changes so that we can confirm the changes you made.
Reply: Authors corrected these changes in the re-revision according to editors’ suggestion.

4) Please upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. We need point-by-point reply to each comment raised by the editor and the reviewers, We are re-sending the comments.
Reply: Ok, We will upload two rebuttle documents addressed by editorial and peer review comments

along with re-revision.

5) Please make sure that your references comply with JoVE instructions for authors. In-text formatting: corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text of the manuscript.
Reply: Authors corrected this in the revision according to editors' suggestion.

6) The highlighted text exceeded JoVE's 2.75 page limit. The editor adjusted the highlighting. Please confirm that you would like to film the highlighted section or change the highlighting to the appropriate section. Make sure you provide all the details and check the protocol for imperative tense.
Reply: Yes, authors agree to editor adjust to the highlighting.

7) Please refer to the previously published articles on JoVE by searching for "morris maze".
Reply: Ok, author do it as editor's suggestion.

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