

Ex-Vivo Analysis Platforms for Amyloid Precursor Protein Cleavage.

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Research Article

Keywords: Alzheimer's disease, Organotypic brain culture, Amyloid β , β -secretase, Hippocampus

Posted Date: July 8th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-474727/v2>

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Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative brain disorder and the most common cause of dementia in the elderly. Large numbers of senile plaques, neurofibrillary tangles, and cerebral atrophy are characteristic features of AD. The main component of senile plaques is amyloid β peptide ($A\beta$), derive from the amyloid precursor protein (APP).

AD has been extensively studied using cell line, primary culture of neural cells, and animal models, however, some discrepancy is observed in these results. Dissociated cultures have lost the tissue architecture of the brain including neural circuits, glial cells, and the extracellular matrix. Animal models require lengthy animal experiments and laborious monitoring of multiple parameters following manipulations. Therefore, it is necessary to connect these experimental models to understand the pathology of AD. In order to analyze long-term neuronal development and plasticity, and progressive neurodegenerative disease, experimental platform amenable to continuous observation and experimental manipulation is required. In this report, we provide a practical method to slice and cultivate rodent hippocampus to consecutively investigate the cleavage of APP and the secretion of $A\beta$ as an ex vivo model.

1. Introduction

Organotypic brain culture, including slice culture, are used to study the central nervous system because they have advantages over both in vivo and in vitro research platforms^{1,2}. Sophisticated cultured brain slices retain tissue structure, neural circuit, and extracellular environment, and replicate the native status of the in vivo context. The slice cultures from hippocampi are widely used to investigate the effect of pharmacological, biochemical, physiological, and genetic manipulation on neuronal and glial cells^{3,4}. Three typical methods are used to maintain neural tissue in vitro, including roller-tube, matrix embedded culture, and membrane-interface⁵. Membrane-interface brain slice culture was improved for practical use by Stoppini et al.⁶. Because sterilized filter membrane cup is designed to easily change the medium, this apparatus as an interface is useful for long-term maintenance of the explant tissue. The cultured hippocampal slices on the filter membrane cup are amenable for completely replacing of culture medium. Previously, we reported about neural circuit, synaptic plasticity, neurodevelopment, and neurodegenerative diseases using cultured hippocampal slices⁷⁻⁹.

Alzheimer's disease (AD) is a chronic neurodegenerative brain disorder that usually starts slowly and worsens over a long period of time, and the most common cause of dementia in the elderly. Numerous genetic and biochemical studies support the hypothesis that an excessive accumulation of amyloid β peptide ($A\beta$) results in aggregation and amyloid deposition in the brains of AD patients (amyloid hypothesis)¹⁰. $A\beta$ derive from the amyloid precursor protein (APP), which an integral type I membrane protein expressed in many tissues and concentrated in the synapses. APP is commonly cleaved by membrane protease in the secretase family, α -, β - and γ -secretase. $A\beta$ is generated by sequential cleavage by β -secretase (BACE1)¹¹ and γ -secretase, whereas alternative cleavage by α -secretase precludes $A\beta$

production¹². Neuronal activity is known as an important regulator of β cleavage of APP^{13,14}. Regulation of cleavage, accumulation, and elimination of A β are explored as a target for therapy and prevention of Alzheimer's disease (AD).

Large amount of data from cell line model-based assay revealed the molecular mechanism of A β secretion to understand AD¹⁰. Although cell line models are powerful tool for molecular biological studies, discordances are observed between cell line and animal models. Because neuronal and glial cell are morphologically and functionally specialized, dissociated primary cultures of neuronal tissue have an advantage to reveal the pathological processes and molecular mechanisms of neuropsychiatric disorder. Although experiments using cultured neuron provide important information, they have lost the tissue architecture of the brain. Neural circuits, glial network, and the extracellular environment are important for neurodevelopment and neurodegenerative disorders, including AD, schizophrenia, and autism spectrum disorder^{11,15-17}. The animal model, however, have disadvantages; they need lengthy animal experiments and substantial cost, and laborious monitoring of multiple parameters following manipulations. Furthermore, genetic engineered animals might be affected by compensatory alterations and developmental changes¹⁸. For the above reason, the hippocampal slice culture may be a potential technique linking between animal model and neural cell.

The hippocampus is thought to be one of the first regions of the brain to suffer damage in AD¹⁹. In this report, we describe methods of organotypic hippocampal slice culture and consecutive analysis of A β and related products, which plays a central role in the pathogenesis of AD. Continuous collection and analysis of A β is a powerful approach to elucidate the cellular and molecular mechanism underlying AD. To develop and evaluate therapeutic agents and approach for AD, long term and consecutive analysis are also required. Here we provide the standard experimental conditions for analyzing of A β production.

2. Material And Methods

2.1. Animal care

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Juntendo University Graduate School of Medicine (Approval No. 1187) and were conducted in compliance with the ARRIVE guidelines.

2.2. Reagents for slice culture

Slice medium contained 50% minimal essential medium based on Hanks' salts (MEM, Nacalai tesque, Kyoto, Japan, 21442-25, 72mM Glucose, 2mM HEPES are added) 25% Hank's Balanced Salt Solution (HBSS, Thermo Fisher Scientific, 24020-117, MA, USA), and 25% Horse serum (HS, Thermo Fisher Scientific, 16050-122, heat-inactivated at 56°C for 30 min). Dissection solution was ice-cold Gey's BSS (137 mM NaCl, 5 mM KCl, 0.18 mM KH₂PO₄, 0.84 mM Na₂HPO₄ 12H₂O, 36 mM glucose, 1.5 mM CaCl₂, 1 mM MgCl₂, 0.32 mM MgSO₄) saturated with O₂. Phosphate-buffered saline (PBS). 4% Paraformaldehyde

in PB (Nacalai 09154-85). α -secretase inhibitor; GI 254023X (Tocris), β -secretase inhibitor; BACE inhibitor IV (Calbiochem). Primary antibody: anti-NeuN antibody (a marker protein for neuronal nuclei; mouse monoclonal, A60, Chemicon, CA, USA, anti-GFAP antibody (a marker protein for astrocyte; mouse monoclonal, SMI22, BioLegend, CA, USA), anti-Iba1 antibody (a marker protein for microglia; rabbit, 019-19741, Wako Pure Chemical Industries, Osaka, Japan), anti-BACE1 (rabbit monoclonal, D10E5, Cell signaling Technology), anti-APP (mouse monoclonal, 22C11, Millipore). Secondary antibody: Goat Anti-mouse IgG (Alexa Fluor 488) A-11001, Goat Anti-mouse IgG (Alexa Fluor 594) A-11032, Goat Anti-rabbit IgG (Alexa Fluor 488) A-11034, Goat Anti-rabbit IgG (Alexa Fluor 594) A-11037 (Thermo Fisher Scientific, MA, USA). RIPA buffer [1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 25 mM Tris-HCl, pH 7.5, 137 mM NaCl, and 3 mM KCl with a protease inhibitor cocktail (Complete, Roche Diagnostics, Mannheim, Germany)]. 1× Tris-buffered saline (TBS: 50 mM Tris, 138 mM NaCl, 2.7 mM KCl). TBST (1× TBS with 0.1% Tween 20). Mouse/rat amyloid β (1–40) assay kit (IBL, 27720), Mouse/rat amyloid β (1–42) assay kit (IBL, 27721), Mouse/Rat sAPP α (highly sensitive) Assay Kit (IBL, 27419), Mouse sAPP β -w Assay Kit (IBL, 27416).

2.3. Apparatus for slice culture

Tissue chopper (McIlwain)

Double Edge Stainless Steel Cutting Blades (FEATHER, FA-10)

Disposable scalpel (FEATHER, No.11, No. 22)

Filter membrane cup (Millicell-CM, Millipore Carrigtwohill, Ireland, PICM03050 or PICM0RG50)

Stereomicroscope (Olympus, SZX7)

Overhead projector (OHP) sheet cut into 5 cm squares.

2.4. Hippocampal slice culture method

The hippocampal slice culture procedure in this study is an improvement of the method in the previous study.^{9 20 21}

Animals were purchased from Nihon SLC (Hamamatsu, Japan) and treated according to our institutional guidelines. Sprague-Dawley rats or ICR mice hippocampal slice cultures were prepared following protocol. Whole brains were excised from CO₂ anaesthetized rats or mice at postnatal day 6–8 and the hippocampi were isolated. The 400 μ m thick hippocampal slice were obtained from the central region of the hippocampi, using a tissue chopper (McIlwain) with a cutting blades. (Fig. 1A). The slices were placed on the center of filter membrane cups, and SCM was added up to the bottom surface of the filter (Fig. 1B). These prepared cultures were maintained at 37°C, 5% CO₂. The culture medium was replaced twice a week with fresh medium during the entire culture period.

2.5. Protocol

This protocol optimizes previous studies for long-term culture of many hippocampal slices^{6,22}

1. Wipe the stereomicroscope, tissue chopper, and micro pipette with 70% ethanol solution. Sterilize the dissecting instruments and a cutting blade by immersing it in a 70% ethanol solution for 30 minutes. In addition, sterilize them for 30 minutes with UV light in the clean bench.
2. Dispense GBSS into sterilized 100 mL bottle and place it on ice-box. Bubble the GBSS with 100% O₂ for 20-30min. 40-50mL GBSS is required per culture session (2–4 pups). Due to the high oxygen demand of the central nervous system, the working solution needs to be sufficiently oxygenated and cooled. Oxygen is blown through a long needle with a syringe filter to aseptically oxygenate the working solution.
3. Place the tissue chopper on clean bench and attach a sterilized blade, and place autoclaved OHP sheet on cutting chamber.
4. Prepare a 6-well plate or 35 mm dishes, add 1 mL of SCM per well, and place a culture insert in each well. Carefully check that the filter membrane of the insert is completely wet and free of air bubbles underneath. Place the plates or dishes with culture inserts maintained at 37°C, 5% CO₂ until needed.
5. The rodent pups are deeply anaesthetized and euthanized by decapitation. Cut the scalp along the midline to expose the skull. Cut along the sagittal suture and remove the skull from the rostral to the caudal. Quickly scoop out the brain with a micro medicine spoon and place it in oxygenated GBSS on ice.
7. Place the brain on an autoclave filter paper soaked in ice-cold oxygenated GBSS in a 100 mm dish and use a scalpel to remove the midbrain and cerebellum, then separate the hemisphere.
6. Under the stereomicroscope; gently remove the thalamus and the hippocampi are then exposed on each hemisphere in ice-cold oxygenated GBSS. Then gently scoop the hippocampus out with cortex using micro spoon (Fig. 1C).
7. Transfer the hippocampus to the OHP sheet on the tissue chopper chamber. Align the hippocampi perpendicular to the blade and drain excess of GBSS and obtain coronal sections (Fig. 1D).
8. Slice the hippocampi every 400 µm using a tissue chopper. Since tissue chopper and OHP sheet are placed at room temperature, quick operation is required in slicing brain.
9. Transfer sliced hippocampi from the OHP sheet to 60 mm dish filled with ice-cold oxygenated GBSS. Since tissue chopper and hippocampi are placed at room temperature, quick operation is required in steps 6 to 9.
10. Under the stereomicroscope (Olympus, SZX7); Pinch the cortex with precision tweezers and divide the undamaged hippocampal slices one by one. And then split into cortex and hippocampus by scalpels (Fig. 1E). It is important to complete the steps 5 to 10 within 45 minutes.
11. Incubate separated hippocampi in new oxygenated GBSS on ice for 30–60 min.
12. Transfer individual slices on to the culture inserts in the six-well plate or 35 mm dish with pre-warmed SCM. Place 1–6 slices on one culture insert, without placing slices near the walls of the inserts or close to each other. (Fig. 1F).
13. Return the plate or dish to incubator and maintain the slices at 37°C with 5% CO₂-enriched humidified atmosphere.

14. Replace with fresh SCM once every 3–4 days during the culture period. Aspirate the medium in dish and add 750–800 μL of pre-warmed fresh SCM per well. Approximate 200–250 μL of medium would be remained in the culture dish and the filter membrane. Submersion of sliced brain in medium damages nerve cells due to oxygen deficiency. To avoid neural cell death, contact the brain slice with both air and medium that exudes from the filter membrane.

2.6. Immunohistochemical staining

For the immunohistochemical staining, the cultured slices were fixed with 4% paraformaldehyde in phosphate-buffered saline for 1 h at 4°C. The fixed preparations were rinsed 4 times with phosphate-buffered saline (PBS) and then treated with PBS containing 0.1% Triton X-100 and 5% fetal bovine or horse serum at 24°C for 30 min. The treated slices were incubated with a primary antibody against NeuN (1:200 dilution), GFAP (1:200), and Iba1 (1:100) at 4°C for 24 h. Slices treated with primary antibody and washed 5 times with PBS were incubated with a secondary antibody conjugated with Alexa Fluor 488 or 594 (1:400 dilution) at 24°C for 2–2.5 h²¹. The slices were examined using an Olympus IX71 (Olympus, Tokyo, Japan) with Orca-ER cooled CCD camera (Hamamatsu Photonics, Hamamatsu, Japan) or a Leica SP5/TCS confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany)²³.

2.7. Electrophoresis and Immunoblotting

Acute or cultured hippocampal slices were solubilized in ice-cold RIPA buffer with a protease inhibitor cocktail (Complete, Roche Diagnostics, Mannheim, Germany). Samples were resolved by SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Immobilon-P, Millipore)⁹. Membranes were incubated in 5% skim milk in TBST at 25 °C for 30–60 min to block nonspecific binding. Membranes were incubated at 4 °C overnight or 25 °C for 2 h with primary antibodies against GFAP (BioLegend), Iba1 (Wako Pure Chemical Industries), anti-galectin-3 (mouse monoclonal, A3A12, Santa Cruz, CA, USA), or anti- β -actin (a loading control, mouse monoclonal; Wako Pure Chemical Industries). After four changes of 1 \times TBST and three 5-min washes at 25 °C, membranes were incubated in horseradish peroxidase-conjugated secondary antibodies. After four washes at 25 °C, membranes were incubated with ECL solution (Thermo Fisher Scientific, MA, USA). For quantification, chemiluminescence light signals in Super Signal Dura substrate (Thermo Fisher Scientific) were captured by a cooled charge-coupled device camera system (LAS-3000plus; Fuji Photo Film Company, Kanagawa, Japan) that ensured wide ranges of linearity. Densitometric quantification of synaptic protein expression normalized to GAPDH or β -actin. The protein content was estimated using bicinchoninic acid (BCA) reagent (Thermo Fisher Scientific)⁹

2.8. Enzyme Immunoassay for Amyloid β and soluble APP.

The levels of A β and sAPP in SCM measured using ELISA kit following a previous report⁹. The levels of A β_{40} were measured using a solid phase by sandwich ELISA kit following the supplier's information (IBL, Gunma, Japan). The test samples and standards were added to the 96 well plate (precoated with anti-human A $\beta_{(35-40)}$ (1A10) mouse IgG) and incubated overnight at 4°C. After several washes with EIA wash buffer, 100 μL of HRP-labeled anti-A $\beta_{(1-16)}$ rabbit IgG solution was added to each well (except the wells

corresponding to reagent blanks) and incubated at 4°C for 1 h. After a thorough wash, 100 µL of tetramethylbenzidine solution was added to each well and incubated in the dark at 24°C for 30 min. The reaction was stopped by adding 100 µL of 1N sulfuric acid, and the readings were taken at 450 nm. The levels of Aβ in the test samples were calculated by incorporating the unknown values into the standard curve obtained in the assay. The levels of soluble amyloid precursor protein β (Aβ₄₂) were measured using the IBL assay kit (IBL, 96 well plate precoated with anti-Aβ₍₃₈₋₄₂₎ rabbit IgG, HRP-labeled anti-Aβ₍₁₋₁₆₎ rabbit IgG Fab) as described above. The levels of soluble APPα and APPβ were measured using the assay kit (IBL, 96 well plate precoated with anti-mouse APP(599) rabbit IgG, HRP-labeled anti-mouse N-APP rabbit IgG Fab) or (IBL, 96 well plate precoated with anti-mouse sAPPβ-w rabbit IgG, HRP-labeled anti-mN-APP rabbit IgG Fab) as described above.

2.9. Drug application

Test drugs were dissolved into water to concentrations 1000 times higher than the final levels, kept at 4 or -20°C until use, and diluted into medium unless otherwise stated. The GI 254023X and BACE inhibitor IV were dissolved into DMSO (Sigma-Aldrich, MI, USA) to concentrations of 500 and 1000 times higher than the final levels, respectively, and kept at -20°C until use. GI 254023X and BACE inhibitor IV were added to fresh SCM before analysis.⁹

2.10. Statistical analysis

All quantitative data were presented as mean ± standard deviation (SD) or standard error of the mean (SEM). Statistical analysis was performed using either Student's t-test (two-group comparison) or ANOVA (more than two groups) followed by post hoc comparison. Statistical analyses were performed with Prism 5.0 (GraphPad Software, Inc., CA, USA). The level of significance was indicated by asterisks: *P < 0.05, **P < 0.01, ***P < 0.001

3. Results

3.1. Chronological analysis of cultured hippocampal slices

Previously, we have reported that cultured rat hippocampal slices retained neuronal activity for several weeks^{7,21}. Furthermore, we recently showed reorganization and maturation of synapses occurred in cultured hippocampal slices⁹. In this report, we investigated morphological feature of neuronal and glial cell in hippocampal slice cultures after 29 days in vitro (DIV) (Fig. 2A-C). Glial fibrillary acidic protein (GFAP) is the hallmark intermediate filament protein in astrocytes²⁴. Ionized calcium-binding adaptor protein-1 (Iba1) is a microglial and macrophage-specific protein²⁵. In slice culture, astroglia and microglia are distributed throughout the slice, and the arrangement of neurons was maintained. Hippocampal subregions such as dentate gyrus (DG), CA1 and CA3 could be clearly discerned (Fig. 2A). Next, to quantitatively evaluate the existence of living cells, we assessed total protein contained in organotypic hippocampal slice cultures by Micro BCA Protein Assay Kit. These data indicate that neural cells remained in cultured hippocampal slices for over 12 weeks (Fig. 2D). Furthermore, we assessed the

amount of neuron, astrocytes and microglia using immunoblotting (Fig. 2E-I). Neurons decreased just after plating the hippocampal slice on a filter membrane cup and stabilize after 7 days. Astrocytes and microglia increased 1 day after plating. Galectin-3, which required for microglia activation in injured brain²⁶, was elevated for 1 week after preparation and then declined.

3.2. Alternative cleavage of APP

Previously, reorganization and maturation of neuron were reported in cultured hippocampal slices^{27,28}. We evaluated the expression level of BACE1, and APP by immunoblotting assays for several weeks. These indicate that BACE1 expression decreased after 4 days, and APP expression were stable during the culture period. (Fig. 3A-C). APP is alternatively cleaved by the members of the α or β secretase family. The α -secretases are members of the ADAM family, and considered to be a part of the non-amyloidogenic pathway in APP processing^{29,30}. We assessed enzyme dependencies on APP metabolism in hippocampal slice culture, using α or β secretase inhibitor (50 μ M GI254023X and/or 2 μ M BACE inhibitor IV). We cultured hippocampal slices with fresh SCM containing vehicle (DMSO) or inhibitors after washing three times with 1 mL fresh SCM for 24 h. In accordance with previous reports³¹, treatment of secretase inhibitors increased immature and mature APP as a result of reduction of APP cleavage. Treatment with both inhibitors blocked almost all the first step of APP cleavage, further increasing the amount of APP full-length APP (Fig. 3D-G).

3.3. Biochemical analysis of production of A β

Since organotypic cultures are artificial environments, we are able to control extracellular conditions including temperature, oxygen content, nutritional factor, and neural activity. To elucidate the optimal condition for analysis of A β secretion, we examined the effect of the number of slices on filter membrane cup and incubation period. Because reducing the number of slices in a cup allows for more efficient experiments, we plated on 1 to 6 slices on a filter membrane cup for culture, and cultured slices were incubated with 1mL fresh SCM for 24h. We measured total protein of slice and A β in SCM from cultured hippocampal slices with a two-site ELISA assay (see materials and methods). Total protein of a cultured hippocampal slice was $52.8 \pm 9.3 \mu\text{g}$. The correlation coefficient between A β_{40} and slice number was $R^2 = 0.9607$, $P < 0.0001$ (Fig. 4A). In accordance with previous reports, single hippocampal slice secreted 75–120 pg A β_{40} for 24h (Fig. 4B). Further analysis showed that the ratio of A β_{40} secretion to total protein amount is 1.7 to 2.8 pg/ μg for 24h (Fig. 4C). There was no significant difference in A β_{40} secretion between groups ($P > 0.05$). For assessing incubation time for A β_{40} assay, we cultured four hippocampal slices for 1 to 90 h with fresh SCM after washing three times with 1mL fresh SCM. A statically significant ($P < 0.0001$) strong correlation ($R^2 = 0.9190$) was observed between A β_{40} secretion and incubation time (Fig. 4D). Our time-lapse analysis of A β secretion showed that single hippocampal slice secreted $2.68 \pm 0.82 \text{ pg}$ for 1h (Fig. 4E).

3.4. Chronological analysis of production of A β

We investigated whether or not the amount of A β secretion changed depending on the culture period. To quantify A β secretion levels, we collected SCM cultured hippocampal slices (1–58 DIV) for 24 h after washing three times with 1 mL fresh SCM. We previously reported that secretion of sAPP β was between 3 to 6 ng/mL, and secretion of A β ₄₀ was between 200 to 300 pg/mL during the monitoring period⁹. The A β ₄₀ and A β ₄₂ are the most common isoform of A β in brain and the main component of the amyloid plaques found in the brains of AD. Here we measure the A β ₄₂ in same SCM preparation using a two-site ELISA assay. Secretion of A β ₄₂ was 23 to 50 pg, and ratio of A β ₄₂ to A β ₄₀ was 10 to 17 % during the monitoring period (Fig. 4F, S1).

3.5. Comparison of species differences in APP cleavage

In order to apply hippocampal slice culture to various research fields, we assessed the amount of A β secretion by comparing rat and mouse. The amount of A β ₄₀ secretion from cultured mice hippocampal slice was 160 ± 15 pg/mL (Fig. 5A). Although A β secreted from mouse hippocampus slices were less than that of rats (350 ± 65 pg/mL), there was no significant difference in A β secretion compared to the total protein amount of slices (mice: 1.75 ± 0.3 pg/ μ g, rats: 1.86 ± 0.3 pg/ μ g, Fig. 5B, C). APP is commonly cleaved by α - or β -secretase both remove and release nearly the entire extracellular domain which called soluble APP (sAPP α , and sAPP β). This alternative cleavage is critical for revealing the molecular mechanism of AD pathology. We revealed that both sAPP α and sAPP β are secreted from hippocampal slice cultures by sAPP assay. The amount of secretion of sAPP α and sAPP β from four cultured rat hippocampal slices were 1.30 ± 0.21 ng and 0.63 ± 0.18 ng. The amount of secretion of sAPP α and sAPP β from four cultured mouse hippocampal slices were 0.69 ± 0.09 ng and 0.44 ± 0.03 ng (Fig. 5D, E). These results indicated that α cleavage was dominant as in the model animal.

4. Discussion

In this report, we showed a method for preparation of hippocampal slice culture and consecutive analysis of A β . The cultured hippocampal slice preserved tissue architecture, neural circuits, and synaptic dynamics^{6,27,28}. Here, we showed that the existence of microglia, astrocyte, and neuron in a cultured slice for several weeks or potentially several months (Fig. 2). Our chronological immunoblotting analysis indicated that glial cells have proliferated for 1 to 2 weeks, and then be maintained for several weeks (Fig. 2G). Besides, galectin-3, which required for microglia activation and proliferation in injured brain²⁶, was elevated for 1 week after preparation and then declined (Fig. 2I). These data may indicate that microglia were activated to eliminate dead or apoptotic cells. The expression levels of synapse marker proteins, synaptophysin and PSD95 decreased from 1 to 4 days, and increased after 7 days⁹. Quantitative analyses suggested that expression levels of APP were not changed significantly, and expression levels of BACE1 were stable after 7 DIV (Fig. 3). Since BACE1 is mainly expressed in neuron, the expression level of BACE1 decreases due to neuronal cell death in the first week of culture (Fig. 2F). Immunoblotting analysis indicated that the molecular weight of BACE1 changed slightly after 4 DIV, which may represent the effect of the BACE1 maturation process. Newly synthesized BACE1 is cleaved at

its prodomain before transport to the trans-Golgi network³². These results indicated that slice culture may be suitable for continuous ex vivo analysis of APP cleavage. These results provide that slices cultured after 2 or 3 weeks are suitable for analysis of neural functions and APP processing.

A β is formed after sequential cleavage of the APP by β - and γ -secretase, accumulates in the central nervous system and subsequently initiates the neural dysfunction. An excessive accumulation of A β results in aggregation and amyloid deposition in the brains of AD patients³³. The γ secretase cleaves within the transmembrane region of APP and can generate a number of isoforms of 30–51 amino acid residues in length³⁴. The A β_{40} and A β_{42} are the most common isoform of A β in brain and the main component of the amyloid plaques found in the brains of AD. The A β_{40} form is the more common of the two, but A β_{42} is the more fibrillogenic and is thus associated with disease states³⁵.

Since the pathological changes of AD are progressing slowly, a model for continuously analyzing central nervous function for a long period is required. In this report, we evaluated the method to investigate A β formation continuously using cultured hippocampal slices. To estimate A β production, we measured A β_{40} and A β_{42} secreted from hippocampal slices using a two-site ELISA assay. In culture medium, four cultured hippocampal slices secreted 200–400 pg A β_{40} and 23–50 pg for 24 h⁹. These data indicated that 2.0–4.0 pg A β_{40} and 0.2–0.5 pg A β_{42} were secreted by a cultured hippocampal slice in 1 h. In accordance with previous reports¹³, the ratio of A β_{42} to A β_{40} was between 10 to 17 % (Fig. 4). As a result of examining the differences between species, there was no difference between the amount of A β_{40} and sAPP secretion in mouse and rat hippocampal slices (Fig. 5).

Here we tried to optimize the incubation period and the amount of slices for analysis of A β . We placed 1 to 6 hippocampal slices on a membrane cup for one week culture, and incubate these slices for 24h with fresh SCM for A β assay. The concentration of A β linearly increases corresponding to the number of slices (Fig. 4). For time-lapse analysis of A β secretion, we cultured four hippocampal slices and incubate for 1 to 90 h with fresh SCM after washing three times with 1 mL fresh SCM. Our results indicate that single hippocampal slice secreted 2.68 ± 0.82 pg for 1h (Fig. 4B, C). The dispersion of A β assay indicates that the carryover medium significantly impacts the measurement results with short term incubation. When many slices are placed on a membrane cup, it is easy to analysis of A β production, but proper placement of slices is difficult. We suggest that it is appropriate to incubate two to six hippocampal slices in a membrane cup.

The α -secretase family alternatively cleaves the APP within the A β sequence. Thus, α -cleavage precludes A β formation and is considered to be part of the non-amyloidogenic pathway in APP processing³⁶. In cultured hippocampal slice, alternative cleavage of APP is conserved (Fig. 3), and thus application of the α -secretase inhibitor increased secretion of A β ⁹.

Synaptic plasticity is activity dependent changes in synaptic function that is thought to play a crucial role in learning and memory³⁷. Two forms of long-term plasticity, long-term depression (LTD) and long-term

potentiation (LTP) are involved in long term memory and induce in acute brain slice. In cultured hippocampal slice, the synaptic plasticity was occurred²⁸, and synapse formation or elimination is induced by repetitive induction of LTP or LTD^{7,8}. Because synaptic plasticity is disturbed in neuropsychiatric disorder model including AD^{38,39}, rodent hippocampal slice culture is a good in vitro experimental model for pathological changes in the central nervous system. Here we showed no difference in A β secretion between mouse and rat hippocampal slice culture (Fig. 5).

Because of individual differences and difficulty of manipulation in animal models, comparative analyses before and after administration in same preparation are not easy. To evaluate drug efficacy, however, this comparative analysis between before and after administration in same cultured preparation is meaningful. Because primary dissociated neural cultures are vulnerable to drug washout during media changes, comparative analysis using the same preparation is extremely difficult in actuality. Filter membrane cups allow easy handling of preparations; therefore, we were able to collect and change culture media without damage and perform comparative analyses using the same preparations (Fig. 4). Because the membrane cup is designed for disposable use, slice culture is a costly method. Using self-made devices and filter membranes can reduce costs⁴⁰.

Slice culture are usually derived from early postnatal or embryonic rodents. Because essential cytoarchitecture are already established but neural circuits are still immature, early postnatal periods (day 0 to 10) are ideally suited for culturing. Some attempts have been made to culture adult tissue of model animal for elucidating age-related neurodegenerative diseases⁴¹.

Slice culture have been used for wide research field ranging from physiology, pharmacology, endocrinology, biochemistry, and development to pathology. We anticipate that hippocampal slice culture will be used to reveal molecular and cellular basis of neuropsychiatric disorder as ex vivo model.

We provide a method of consecutive analysis protein secretion from brain slices to study neurodegenerative disease. Hippocampal slice culture is an experimental system that effectively keeps the in vivo neuronal network and seems to be a convenient method to study the cellular and molecular mechanism underlying the neuropsychiatric disorders including AD, and to evaluate therapeutic approach for such diseases.

Declarations

Ethics approval and consent to participate:

All the biosafety and approvals for the ethical and humane use of animals were obtained prior to the start of the study. All procedures performed in this study were in accordance with the ethical standards of Juntendo University. This study did not involve human participants.

Consent for publication:

Not applicable.

Availability of data and materials:

Original raw data are available from Juntendo University (Department of Pharmacology) and can be readily furnished upon request.

Funding:

This work was supported by JSPS KAKENHI Grant Number JP20K07765 and JP17K09040 (to Y.K), and grants-in-aid from the TERUMO LIFE SCIENCE FOUNDATION (to Y.K).

Authors' contributions:

Y.K. conceived and designed the study. Y.K, H.J, K.N, Y.H and N.T. contributed to investigation and data analysis. Y.K. wrote and reviewed the manuscript. Y.K. and T.S. supervised the study. All authors approved the final version of the manuscript.

Acknowledgements:

We would like to thank the Laboratory of Proteomics and Biomolecular Science, Morphology and Image Analytics, and Biomedical Research Resources, Research Support Center, Juntendo University Graduate School of Medicine for technical assistance.

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Figures

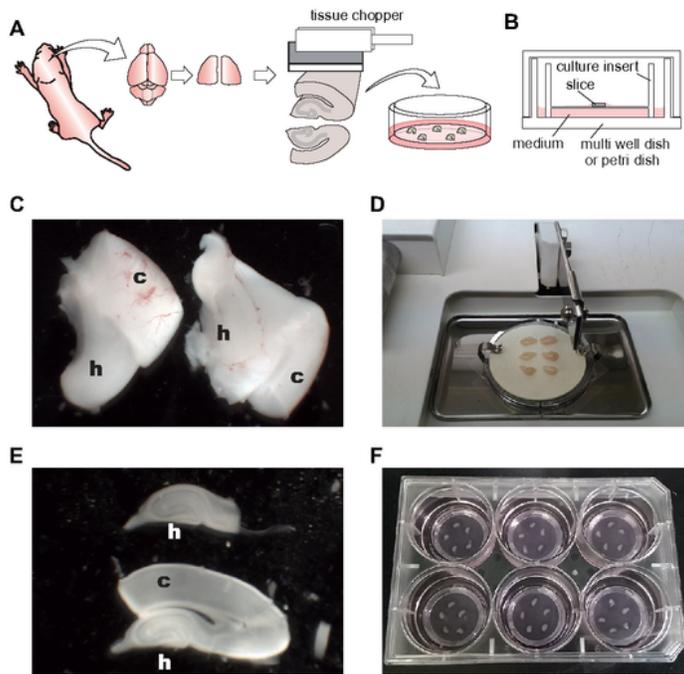


Figure 1

Schematic diagrams and photographs of procedure for hippocampal slice culture. (A) Shema of hippocampal slices culture method. (B) Scheme representing in transverse section of disposition of slice on membrane culture insert and within the culture dish. (C) Image of isolated hippocampi (h) with cerebral cortex (c). (D) Image of tissue chopper and isolated hippocampi with cerebral cortex. Six hippocampi were placed on OHP sheet in cutting chamber. (E) Sliced hippocampus (h) with cortex (c) and

isolated hippocampus. (F) Five slices were placed on to the filter membrane cup in the six-well plate with SCM. Hippocampal slices are not located close to the cup walls of the inserts or close to each other. Scale indicates 50 μm .

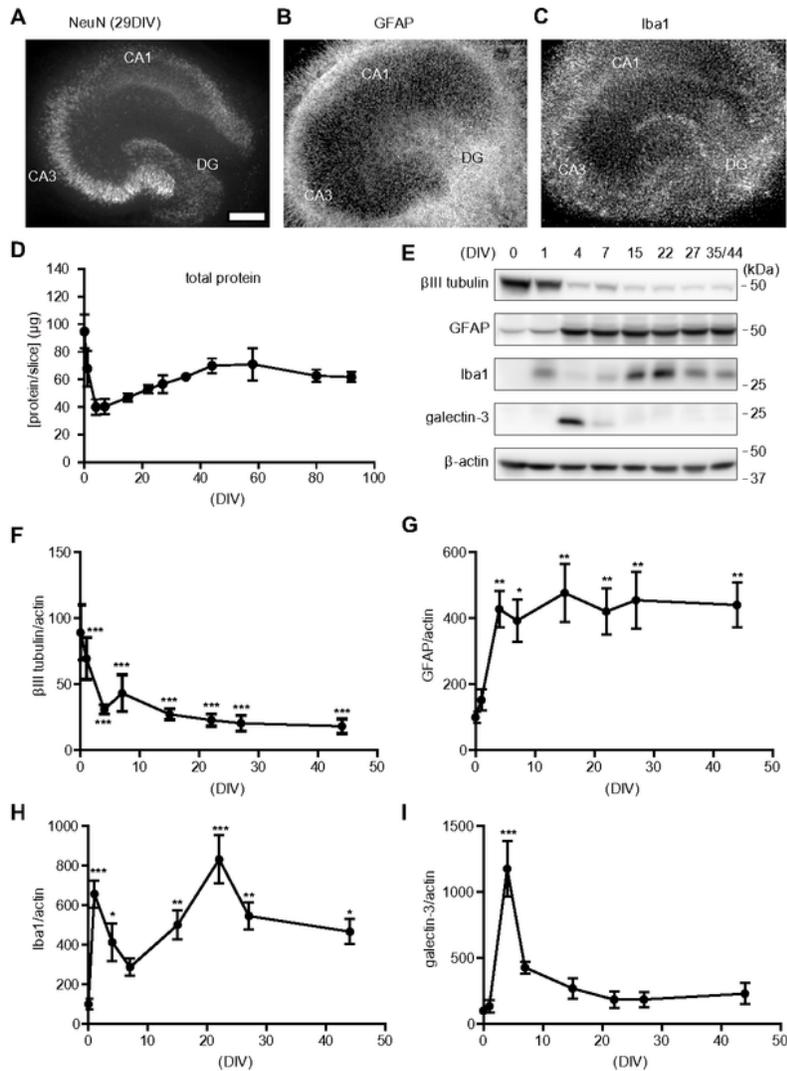


Fig.2 Kamikubo et al.

Figure 2

Chronological analysis of neuronal cells in cultured hippocampal slices.. (A-C) Immunofluorescence staining of cultured hippocampal slices antibodies against NeuN (A), GFAP (B), and Iba1 (C). CA1; Cornu

Ammonis 1, CA3; Cornu Ammonis 3, DG; dentate gyrus. (D) Quantitative analysis of total protein in the lysate from four acute slices (0 DIV) or cultured slices (1-90 DIV). (E-I) Immunoblotting analysis of hippocampal slices with antibodies against β III tubulin (F), GFAP (G), Iba1 (H), and galectin-3 (I), normalized to β -actin served as a loading control. Data were analyzed by one-way ANOVA, and differences between pairs were analyzed using Dunnett's multiple comparison post tests (versus 0 DIV). NS, $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Data are mean \pm SEM, based on four independent experiments ($n = 4$).

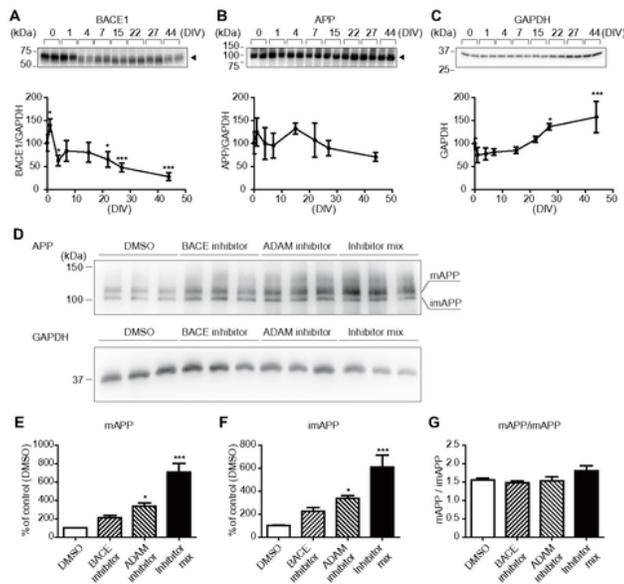


Fig.3 Kamikubo et al.

Figure 3

Chronological analysis of APP and synaptic protein by immunoblotting. Immunoblotting analysis of hippocampal slices (0-44 DIV) with antibodies against BACE1 (A), APP (B), and GAPDH (C). Each point represents a percentage of the 0 DIV (acute slices) signal. Data were analyzed by one-way ANOVA, and differences between pairs were analyzed using Dunnett's multiple comparison post tests (vs. 0 DIV). NS, $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Data are mean \pm SD, based on four independent experiments ($n = 4$). (D-G) Immunoblotting analysis of APP cleavage. Cultured hippocampal slices ($n = 3$) treated with ADAM inhibitor (GI254023X, 50 μ M) and/or BACE1 inhibitor (BACE inhibitor IV, 2 μ M) for 24 h. Data were analyzed by one-way ANOVA, and differences between pairs were analyzed using Dunnett's multiple comparison post tests (vs. DMSO). NS, $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Data are mean \pm SEM, based on three independent experiments ($n = 3$).

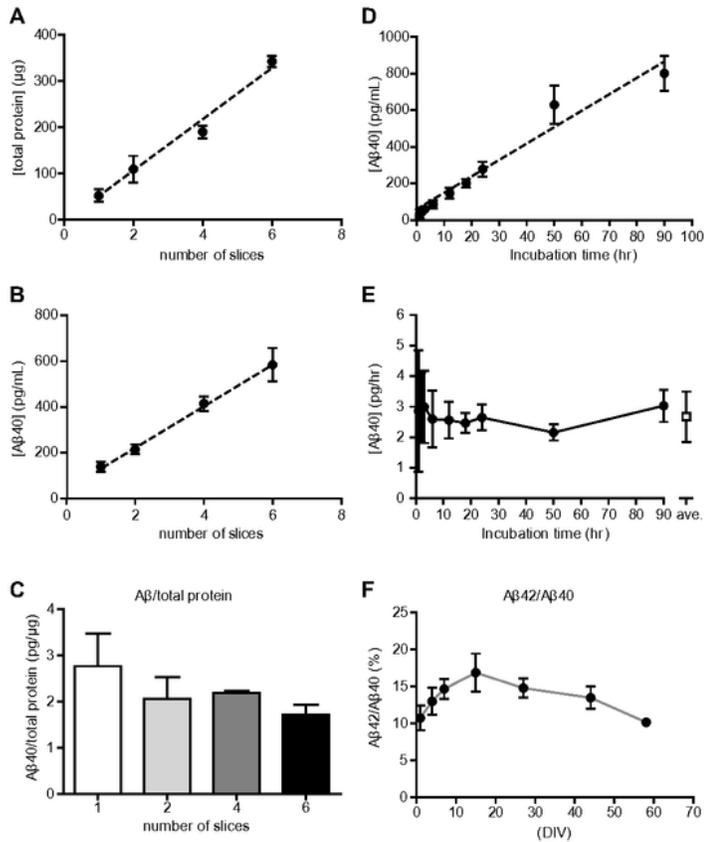


Fig.4 Kamikubo et al.

Figure 4

Culture condition and period dependency of the secretion of Aβ40. (A) Total protein in the lysate from 1, 2, 4, or 6 hippocampal slices. $R^2 = 0.9623$, $P < 0.0001$. (B) Quantification of Aβ40 secreted from 1, 2, 4, or 6 cultured hippocampal slices (15 DIV) for 24hr. Results are mean \pm SD based on three independent experiments ($n=3$). $R^2 = 0.9607$, $P < 0.0001$. (C) The ratio of Aβ40 to total slice protein. Statistical significance was tested by one-way ANOVA, and pairwise comparison was performed according to Tukey-

Kramer multiple comparison test. There was no significant difference between groups, $P > 0.05$. (D) Quantification of A β 40 secreted from a cultured hippocampal slice (15-21DIV) for 1-90 hr. Results are mean \pm SD based on four (1, 2, 3, 6, 12, 18 h) or eight (24, 50, 90 h) independent experiments (n=4 or 8). $R^2=0.9190$, $P < 0.0001$. (E) A β 40 secretion from a cultured hippocampal slice per 1h. The amount of A β 40 secretion is compensated for residual medium (filled circle). Open square; an average of all data (2.68 ± 0.82 pg/hr, n=48, 1-90 h). (F) Chronological quantitative analysis the ratio of A β 42 to A β 40 in SCM. Four hippocampal slices plated on a Millicell culture insert. For A β assay, hippocampal slices cultured with fresh SCM for 24h at 37 °C. A β 40 and A β 42 were quantified by ELISA.

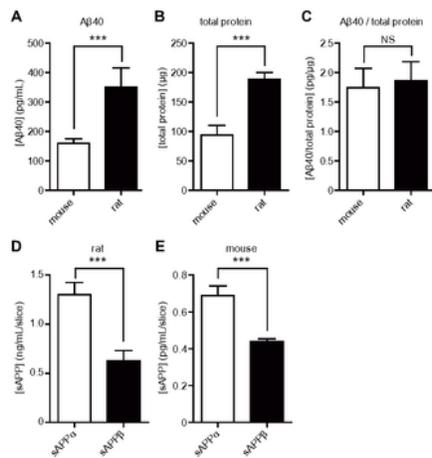


Fig.5 Kamikubo et al.

Figure 5

APP cleavage products from cultured mouse and rat hippocampal slices. Aβ40, sAPPα, and sAPPβ in SCM were quantified by ELISA. Four hippocampal slices prepared from rat or mouse plated on a filter membrane cup. (A) Quantitation of Aβ40 secreted from hippocampal slices cultured with fresh SCM for 24h at 37 °C. (B) Total protein of rat or mouse hippocampal slices. (C) The calculated data of the ratio of Aβ40 to total protein. (D and E) Quantitation of APPα, and sAPPβ secreted from hippocampal slices

cultured with fresh SCM for 24h at 37 °C. Data are mean \pm SD, based on three independent cultures (n = 3). The statistical significance was determined using unpaired t-test. “***”, P < 0.001, “NS”, P > 0.05

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