Ex-vivo analysis platforms for amyloid precursor protein cleavage

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Abstract

Neurodegeneration is the progressive disruption of function or structure of neuronal network in central and peripheral nerve system. Generally, neurodegenerative disease including amyotrophic lateral sclerosis, Parkinson's disease, Huntington's disease, and Alzheimer's disease (AD) occur as a result of neurodegenerative processes. AD is 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. Therefore, it is necessary to connect these experimental models to understand 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 hippocampal to consecutively investigate the cleavage of APP and the secretion of $A\beta$.

1. Introduction

Organotypic brain culture, including slice culture are used in central nervous system research because they have advantages over both in vivo and in vitro platforms [1, 2]. Cultured brain slices preserve 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, physiological, and genetic manipulation on neurons and glia [3, 4]. Three typical methods are used to maintain brain slices in vitro, including roller-tube, membrane-interface, and matrix embedded culture [5]. Membrane-interface neural tissue culture was improved for practical use by Stoppini et al. [6]. Since filter membrane cup is designed to easily change the medium, this apparatus as an interface is convenient 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, development, and Alzheimer's disease (AD) using cultured hippocampal slices [7–9].

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 $A\beta$ production [12]. Neuronal activity is known as an important regulator of β cleavage of APP [13, 14]. Regulation of cleavage, accumulation and elimination of $A\beta$ 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 AD [10]. 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 changes[18].

Organotypic culture is a platform amenable to simultaneous and continuous analysis of A β secretion and neural function.

observation and experimental manipulation is required.

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 AD [19]. 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. Reagents for slice culture

Slice medium contained 50% MEM (Nacalai tesque, Kyoto, Japan, 21442-25, 72mM Glucose, 2mM HEPES are added) 25% HBSS (Thermo Fisher Scientific, 24020-117,MA, USA), and 25% Horse serum (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). 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),anit-Iba1 antibody (a marker protein for microglia; rabbit, 019-19741, Wako Pure Chemical Industries, Osaka, Japan). 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).

2.2. Hippocampal slice culture method

The experiments in this study were approved by the committees on animal experiments of Juntendo University. Animals were purchased from Nihon SLC (Hamamatsu, Japan) and treated according to our institutional guidelines. Rat or mouse hippocampal slice cultures were prepared following protocol. Whole brains were excised from anaesthetized Sprague-Dawley rats or ICR mice at postnatal day 7–8 and the hippocampi were isolated. Slices of 400- μ m thickness were obtained from the central region of the hippocampi, using a tissue chopper (McIlwain). The slices were placed on a polytetrafluoroethylene membrane filter (Millicell-CM, Millipore), and culture medium (see below for composition) was added up to the bottom surface of the filter. These prepared cultures were maintained at 37°C with 5% CO₂-entiched humidified atmosphere. The slice culture medium (SCM) contained 50% minimal essential medium based on Hanks' salts (Nacalai), 25% Hank's balanced salt solution (Nacalai), and 25% heat-inactivated horse serum (Life Technologies, Gaithersburg, MD). The culture medium was replaced twice a week with fresh medium during the entire culture period.

2.3. Protocol

1. Wipe the tissue chopper, a new blade, stereomicroscope, tissue chopper, and dissecting instruments with 70% ethanol solution. 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).
3. Prepare the tissue chopper placing on clean bench and mounting a sterilized blade, and placing autoclaved overhead projector (OHP) sheet, cut into 5 cm squares, on cutting chamber.
4. Prepare six well plates. Add 1 mL slice culture media (SCM) per well and place culture inserts in each well. Make sure the filter membranes are thoroughly wet with no bubbles underneath. Place the plates with filter membrane cups maintained at 37°C with 5% CO₂-entiched humidified atmosphere until needed.
5. The rodent pups are deeply anaesthetized and then killed by decapitation. Cut the scalp and expose the skull. Remove the skull by cutting along the sagittal suture and then from rostral to caudal side. Scoop out the brain quickly with a micro medicine spoon and place it in ice-cold oxygenated GBSS. Place the

brain on autoclaved filter paper immersed in ice-cold oxygenated GBSS on 100mm dish. Use a scalpel to separate the hemispheres leaving out the midbrain.

6. Under the stereomicroscope; after the gently scoop the thalamus, 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 to obtain coronal sections and drain excess of GBSS (Fig. 1D).

8. Slice the hippocampi every 400 μm using tissue chopper.

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 8.

10. Under the stereomicroscope (Olympus, SZX7); separate well defined and undamaged slices from damaged slices, and pinch the cortex with precision tweezers and divide the 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 ice-cold oxygenated GBSS for 30–60 min.

12. Transfer individual slices on to the filter membrane in the six-well plate with SCM. Place 1–6 slices per membrane, not to place the slices either close to the insert wall or close to each other (Fig. 1F).

13. Move dish back to incubator and prepared slices maintained at 37°C with 5% CO₂-entiched humidified atmosphere.

14. The culture medium was replaced twice a week with fresh medium during the entire culture period. Aspirate the medium in dish, and add 750 μL of fresh pre-warmed medium per well. Approximate 250 μL of medium would be remained in the culture dish and the filter membrane.

2.4. Immunohistochemical staining

For the immunohistochemical staining of presynaptic structures, the cultured slices were fixed with 4% paraformaldehyde in phosphate-buffered saline for 1 h at 4°C. The fixed preparations were rinsed 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, 24 h, 4°C), GFAP (1:200 dilution, 24 h, 4°C), and Iba1 (1:100 dilution, 24 h). The primary antibody treated slices were incubated with a secondary antibody conjugated with Alexa Fluor 488 or 594 at 24°C for 2–2.5 h. The slices were examined using a Leica SP5/TCS confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) or an Olympus IX71 (Olympus, Tokyo, Japan) with Orca-ER cooled CCD camera (Hamamatsu Photonics, Hamamatsu, Japan).

2.5. 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). 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× 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).

2.6. Enzyme Immunoassay for Amyloid β and soluble APP.

The levels of $A\beta_{40}$ 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 μ L 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 μ L 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 μ L of 1N sulfuric acid, and the readings were taken at 450 nm. The levels of $A\beta$ 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 ($A\beta_{42}$) were measured using the IBL assay kit (IBL, 96 well plate precoated with anti- $A\beta_{(38-42)}$ rabbit IgG, HRP-labeled anti- $A\beta_{(1-16)}$ rabbit IgG Fab) as described above. The levels of soluble APP α and APP β 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 sAPP β -w rabbit IgG, HRP-labeled anti-mN-APP rabbit IgG Fab) as described above.

2.7. Statistical analysis

All quantitative data were presented as mean $\pm$ 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, 20]. Furthermore, we recently showed reorganization and maturation of synapses occurred in cultured hippocampal slices [9]. In this report, we investigated morphological feature of neuronal and glial cell in cultured hippocampal slice (Fig. 2A-C). Glial fibrillary acidic protein (GFAP) is the hallmark intermediate filament protein in astrocytes [21]. Ionized calcium-binding adaptor protein-1 (Iba1) is a microglial and macrophage-specific protein [22]. Next, to quantitatively evaluate the existence of neural 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 astrocytes and microglia using immunoblotting (Fig. 2E-H). Astrocytes and microglia increased 1 day after plating on filter membrane cups. Galectin-3, which required for microglia activation in injured brain [23], 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 [24, 25]. We evaluated the expression level of BACE1, and APP by immunoblotting assays for several weeks. Our data indicated that expression of BACE1 and APP were stable after two weeks (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 [26, 27]. We assessed enzyme dependencies on APP metabolism in hippocampal slice culture, using each secretase inhibitor. 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 reports [28], 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 1 mL fresh SCM for 24h. We measured A β in SCM from cultured hippocampal slices with a two-site ELISA assay (see materials and methods). For quantitative analysis, we collected SCM and performed A β ₄₀ assay. In accordance with previous reports, single hippocampal slice secreted 75–120 pg

A β ₄₀ for 24h (Fig. 4A). Further analysis showed that A β ₄₀ secretion per total protein amount is 1.7 to 2.8 pg/ μ g for 24h (Fig. 4B, C). 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. Our time-lapse analysis of A β secretion showed that the secretion amount increased linearly up to 90h, and single hippocampal slice secreted 2.68 ± 0.82 pg for 1h (Fig. 4D, E).

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 period [9]. 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, 24, 25]. 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. Besides, galectin-3, which required for microglia activation and proliferation in injured brain [23], was elevated for 1 week after preparation and then declined (Fig. 2H). The microglia were activated for scavenging dead or apoptotic cells. Quantitative immunoblotting analyses suggested that expression

levels of BACE1 and APP were not changed significantly after 2 weeks. The expression levels of synapse marker proteins, synaptophysin and PSD95 decreased from 1 to 4 days, and increased after 7 days [9]. These results provide that slices cultured after 2 or 3 weeks are suitable for analysis of neural functions.

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 patients [29]. The γ secretase cleaves within the transmembrane region of APP and can generate a number of isoforms of 30–51 amino acid residues in length [30]. 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 states [31].

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 h [9]. 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 reports [13], 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 processing [32]. 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 memory [33]. 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 occurred [25], 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 [34, 35], 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 costs [36].

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 diseases [37].

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.

5. Conclusions

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.

Competing interests:

The authors declare that they have no conflict of interests.

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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.

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References

1. Cho S, Wood A, Bowlby MR (2007) Brain slices as models for neurodegenerative disease and screening platforms to identify novel therapeutics. *Curr Neuropharmacol* 5(1):19–33
2. Uesaka N, Abe M, Konno K, Yamazaki M, Sakoori K, Watanabe T, Kao TH, Mikuni T, Watanabe M, Sakimura K et al (2018) Retrograde Signaling from Progranulin to Sort1 Counteracts Synapse Elimination in the Developing Cerebellum. *Neuron* 97(4):796–805.e795
3. Bergold PJ, Casaccia-Bonnel P, Zeng XL, Federoff HJ (1993) Transsynaptic neuronal loss induced in hippocampal slice cultures by a herpes simplex virus vector expressing the GluR6 subunit of the kainate receptor. *Proc Natl Acad Sci U S A* 90(13):6165–6169
4. Ikegaya Y (1999) Abnormal targeting of developing hippocampal mossy fibers after epileptiform activities via L-type Ca²⁺ channel activation in vitro. *J Neurosci* 19(2):802–812
5. Gähwiler BH, Capogna M, Debanne D, McKinney RA, Thompson SM (1997) Organotypic slice cultures: a technique has come of age. *Trends Neurosci* 20(10):471–477

6. Stoppini L, Buchs PA, Muller D (1991) A simple method for organotypic cultures of nervous tissue. *J Neurosci Methods* 37(2):173–182
7. Shinoda Y, Kamikubo Y, Egashira Y, Tominaga-Yoshino K, Ogura A (2005) Repetition of mGluR-dependent LTD causes slowly developing persistent reduction in synaptic strength accompanied by synapse elimination. *Brain Res* 1042(1):99–107
8. Tominaga-Yoshino K, Kondo S, Tamotsu S, Ogura A (2002) Repetitive activation of protein kinase A induces slow and persistent potentiation associated with synaptogenesis in cultured hippocampus. *Neurosci Res* 44(4):357–367
9. Kamikubo Y, Takasugi N, Niisato K, Hashimoto Y, Sakurai T (2017) Consecutive Analysis of BACE1 Function on Developing and Developed Neuronal Cells. *J Alzheimers Dis* 56(2):641–653
10. Hardy J, Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* 297(5580):353–356
11. Vassar R, Bennett BD, Babu-Khan S, Kahn S, Mendiaz EA, Denis P, Teplow DB, Ross S, Amarante P, Loeloff R et al (1999) Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* 286(5440):735–741
12. De Strooper B, Annaert W (2000) Proteolytic processing and cell biological functions of the amyloid precursor protein. *J Cell Sci* 113(Pt 11):1857–1870
13. Kamenetz F, Tomita T, Hsieh H, Seabrook G, Borchelt D, Iwatsubo T, Sisodia S, Malinow R (2003) APP processing and synaptic function. *Neuron* 37(6):925–937
14. Sakurai T, Kaneko K, Okuno M, Wada K, Kashiwama T, Shimizu H, Akagi T, Hashikawa T, Nukina N (2008) Membrane microdomain switching: a regulatory mechanism of amyloid precursor protein processing. *J Cell Biol* 183(2):339–352
15. Sethi MK, Zaia J (2017) Extracellular matrix proteomics in schizophrenia and Alzheimer's disease. *Anal Bioanal Chem* 409(2):379–394
16. Irie F, Badie-Mahdavi H, Yamaguchi Y (2012) Autism-like socio-communicative deficits and stereotypies in mice lacking heparan sulfate. *Proc Natl Acad Sci U S A* 109(13):5052–5056
17. Ichikawa-Tomikawa N, Ogawa J, Douet V, Xu Z, Kamikubo Y, Sakurai T, Kohsaka S, Chiba H, Hattori N, Yamada Y et al (2012) Laminin $\alpha 1$ is essential for mouse cerebellar development. *Matrix Biol* 31(1):17–28
18. Kreiner G (2015) Compensatory mechanisms in genetic models of neurodegeneration: are the mice better than humans? *Front Cell Neurosci* 9:56
19. Hyman BT, Van Hoesen GW, Damasio AR, Barnes CL (1984) Alzheimer's disease: cell-specific pathology isolates the hippocampal formation. *Science* 225(4667):1168–1170
20. Kamikubo Y, Egashira Y, Tanaka T, Shinoda Y, Tominaga-Yoshino K, Ogura A (2006) Long-lasting synaptic loss after repeated induction of LTD: independence to the means of LTD induction. *Eur J Neurosci* 24(6):1606–1616

21. Hol EM, Pekny M (2015) Glial fibrillary acidic protein (GFAP) and the astrocyte intermediate filament system in diseases of the central nervous system. *Curr Opin Cell Biol* 32:121–130
22. Imai Y, Ibata I, Ito D, Ohsawa K, Kohsaka S (1996) A novel gene iba1 in the major histocompatibility complex class III region encoding an EF hand protein expressed in a monocytic lineage. *Biochem Biophys Res Commun* 224(3):855–862
23. Lalancette-Hébert M, Swarup V, Beaulieu JM, Bohacek I, Abdelhamid E, Weng YC, Sato S, Kriz J (2012) Galectin-3 is required for resident microglia activation and proliferation in response to ischemic injury. *J Neurosci* 32(30):10383–10395
24. Buchs PA, Stoppini L, Muller D (1993) Structural modifications associated with synaptic development in area CA1 of rat hippocampal organotypic cultures. *Brain Res Dev Brain Res* 71(1):81–91
25. Muller D, Buchs PA, Stoppini L (1993) Time course of synaptic development in hippocampal organotypic cultures. *Brain Res Dev Brain Res* 71(1):93–100
26. Hardy JA, Higgins GA (1992) Alzheimer's disease: the amyloid cascade hypothesis. *Science* 256(5054):184–185
27. Selkoe DJ (1991) The molecular pathology of Alzheimer's disease. *Neuron* 6(4):487–498
28. Stachel SJ, Coburn CA, Steele TG, Jones KG, Loutzenhiser EF, Gregro AR, Rajapakse HA, Lai MT, Crouthamel MC, Xu M et al (2004) Structure-based design of potent and selective cell-permeable inhibitors of human beta-secretase (BACE-1). *J Med Chem* 47(26):6447–6450
29. Blennow K, de Leon MJ, Zetterberg H (2006) Alzheimer's disease. *Lancet* 368(9533):387–403
30. Olsson F, Schmidt S, Althoff V, Munter LM, Jin S, Rosqvist S, Lendahl U, Multhaup G, Lundkvist J (2014) Characterization of intermediate steps in amyloid beta (A β) production under near-native conditions. *J Biol Chem* 289(3):1540–1550
31. Yin YI, Bassit B, Zhu L, Yang X, Wang C, Li YM (2007) {gamma}-Secretase Substrate Concentration Modulates the Abeta42/Abeta40 Ratio: IMPLICATIONS FOR ALZHEIMER DISEASE. *J Biol Chem* 282(32):23639–23644
32. Lammich S, Kojro E, Postina R, Gilbert S, Pfeiffer R, Jasionowski M, Haass C, Fahrenholz F (1999) Constitutive and regulated alpha-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease. *Proc Natl Acad Sci U S A* 96(7):3922–3927
33. Bliss TV, Collingridge GL (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* 361(6407):31–39
34. Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, Anwyl R, Wolfe MS, Rowan MJ, Selkoe DJ (2002) Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. *Nature* 416(6880):535–539
35. Klyubin I, Walsh DM, Lemere CA, Cullen WK, Shankar GM, Betts V, Spooner ET, Jiang L, Anwyl R, Selkoe DJ et al (2005) Amyloid beta protein immunotherapy neutralizes Abeta oligomers that disrupt synaptic plasticity in vivo. *Nat Med* 11(5):556–561

36. Koyama R, Muramatsu R, Sasaki T, Kimura R, Ueyama C, Tamura M, Tamura N, Ichikawa J, Takahashi N, Usami A et al (2007) A low-cost method for brain slice cultures. J Pharmacol Sci 104(2):191–194
37. Mewes A, Franke H, Singer D (2012) Organotypic brain slice cultures of adult transgenic P301S mice—a model for tauopathy studies. PLoS One 7(9):e45017

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.

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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). (D) Quantitative analysis of total protein in the lysate from four acute slices (0 DIV) or cultured slices (1-90 DIV). (E-H) Immunoblotting analysis of hippocampal slices with antibodies against GFAP (F), Iba1 G),

and galectin-3 (H), normalized to β -actin served as a loading control. Each point represents a percentage of the 0 DIV signal. Data are mean $\pm$ SEM, based on four independent experiments (n = 4).

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Figure 3

Chronological analysis of APP and synaptic protein by immunoblotting. (A-C) Protein lysate from four acute slices (0 DIV) or cultured slices (1-44 DIV) was loaded in each lane. Slices were lysed in RIPA buffer. Immunoblotting analysis of hippocampal slices with antibodies against BACE1 (A), APP (B), and GAPDH (C). Each point represents a percentage of the 0 DIV signal. 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 inhibitor of ADAM and/or BACE.

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Figure 4

Culture condition and period dependency of the secretion of Aβ40. (A) Quantification of Aβ40 secreted from 1, 2, 4, or 6 cultured hippocampal slices (15 DIV) for 24hr. Results are mean ± SD based on three independent experiments (n=3). (B) Total protein in the lysate from 1, 2, 4, or 6 hippocampal slices. (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. (D) Quantification of Aβ40 secreted from a cultured hippocampal slice (15-21DIV) for 1-90 hr. Results are mean ± SD based on four (1, 2, 3, 6, 12, 18 h) or eight (24, 50, 90

h) independent experiments (n=4 or 8). (E) Aβ40 secretion per 1h from a cultured hippocampal slice. The amount of Aβ40 secretion is compensated for residual medium. Open square; an average of all date (2.68 ±0.82 pg/hr, n=48, 1-90 h). (F) Chronological quantitative analysis the ratio of Aβ42 to Aβ40 in SCM. Four hippocampal slices plated on a Millicell culture insert. For Aβ assay, hippocampal slices cultured with fresh SCM for 24h at 37 °C. Aβ40 and Aβ42 were quantified by ELISA.

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Figure 5

APP cleavage products from cultured mouse and rat hippocampal slices. A β 40, sAPP α , and sAPP β in SCM were quantified by ELISA. Four hippocampal slices prepared from rat or mouse plated on a filter membrane cup. (A) Quantitation of A β 40 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 A β 40 to total protein. (D and E) Quantitation of APP α , and sAPP β 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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