Maturational Characterization of Mouse Cortical Neurons Three-Dimensionally Cultured in Functional Polymer FP001-Containing Medium

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The aim of the present study is to construct and characterize a novel three-dimensional culture system for mouse neurons using the functional polymer, FP001. Stereoscopically extended neurites were found in primary mouse cortical neurons cultured in the FP001-containing medium. Neurons cultured with FP001 were distributed throughout the medium of the observation range whereas neurons cultured without FP001 were distributed only on the bottom of the dish. These results demonstrated that neurons can be three-dimensionally cultured using the FP001-containing medium. The mRNA expression of the glutamatergic neuronal marker vesicular glutamate transporter 1 in neurons cultured in the FP001-containing medium were higher than that in neurons cultured in the FP001-free medium. Expression of the mature neuronal marker, microtubule-associated protein 2 (MAP2) a,b, and the synapse formation marker, Synapsin I, in neurons cultured with FP001 was also higher than that in neurons cultured without FP001. The expression pattern of MAP2a,b in neurons cultured with FP001, but not that in neurons cultured without FP001, was similar to that in the embryonic cerebral cortex. Exposure to glutamate significantly increased 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyldiazotoluol bromide (MTT) reduction activity in neurons cultured with FP001 compared to that in neurons cultured without FP001. These results suggested that glutamatergic neurotransmission in neurons three-dimensionally cultured in the FP001-containing medium may be upregulated compared to neurons two-dimensionally cultured in the FP001-free medium. Thus, neurons with the properties close to those in the embryonic brain could be obtained by three-dimensionally culturing neurons using FP001, compared to two-dimensional culture with a conventional adhesion method.

Key words neuron; three-dimensional culture; neurotransmitter; maturation; glutamate

INTRODUCTION

Neurons form complicated neuronal networks that three-dimensionally spread in the living brain, and thereby, perform neurotransmission that is required to exert normal brain function. Therefore, abnormalities in the formation of neuronal networks may result in the onset and/or development of various neuropsychiatric disorders, including depression and schizophrenia. Neurons differentiated from neural stem cells extend neurites and make synapses with other neurons to form neuronal networks. Such neuronal differentiation and maturation requires scaffolds, which include supporting cells, such as glial cells, in the brain. For research purposes, neurons are usually cultured by adhering them on a dish as an in vitro experimental system for analysis of neuronal function. However, neurons in the adhesion culture in dishes only extend neurites in the planar direction, but are not able to reproduce the properties of neurons inside the brain under a stereoscopic environment. Therefore, three-dimensionally cultured neurons have recently been recognized as an alternative tool to analyze more physiologically relevant events in the neuronal networks.

In general, the three-dimensional culture methods are classified into at least three types: Sphere method refers to culturing of aggregated clusters of floating cells, microcarrier-capsule method refers to culturing of cells adhered to particles dispersed in the medium, and embedding method refers to culturing of cells embedded into gels or microfibers as scaffolds. The sphere method is useful for analysis of biological activity of compounds since it is easy to add compounds into the medium of the floating aggregated cells, but may not be suitable for culturing neurons that extend neurites and form neuronal networks owing to the difficulty in getting scaffolds. The microcarrier-capsule method may be suitable for culturing neuronal stem/progenitor cells since a large number of cells can be cultured with a small volume of the medium due to a large surface area of scaffolds with high density of the particles. However, small size of the particles may be insufficient as scaffolds for neurites, and therefore, it may also be unsuitable for culturing neurons. Thus, embedding method has been widely used for three-dimensional culture of neurons to acquire scaffolds for neurite outgrowth.

Puschmann et al. reported three-dimensional culturing of neurons embedded in polyurethane nanofibers, the structure of which is biologically compatible for neurons and suitable for maintaining the three-dimensional structure of neurites and neuronal networks. In addition, embedding method, using

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porous polystyrene and collagen gel as scaffolds, has been reported to be suitable not only for the three-dimensional culture of neurons, but also immunostaining and patch clamping by preparing sliced sections from the embedded cultures. However, the electric current observed in the patch clamp in this three-dimensionally culture of neurons was more similar to that in a section of the living brain, compared to that observed in two-dimensionally cultured neurons. However, embedding culture method may not be suitable for assessing biological activity of compounds due to difficulty in adding or removing compounds which may not be evenly dispersed in the medium, and in collecting cells from embedded gels or fibers.

The FCE medium contains the functional polymer, FP001, which is polysaccharide gellan gum, has a double helix structure under cooling conditions, and forms a bridging structure via carboxyl groups in the presence of divalent ions. By forming a cross-linked structure in the medium, cells can be evenly dispersed and suspended. Therefore, spontaneous aggregation of the cells is reduced, and a uniform size of spheres is obtained during the culture in the FCE medium. In fact, stable large-scale culture of human induced pluripotent stem cells has been reported using the FCE medium. Since the cross-linked structure of FP001 has high fluidity, and the viscosity of the FCE medium is almost equal to that of water, compounds can be freely added and dispersed into the FCE medium. Thus, this medium may be suitable for three-dimensional culture of neurons for the analysis of biological activity of exogenously added compounds. However, there have been very few studies that report such application of the FCE medium to culture neurons.

In the present study, we attempted to observe whether neurons derived from embryonic mouse cerebral cortex can be three-dimensionally cultured in the FCE medium. In this experiment, two types of media commonly used for neuronal culture were used. Furthermore, by comparing the expression of various neuronal markers between the neurons three-dimensionally cultured in the medium with FP001 and the neurons two-dimensionally cultured in the medium without FP001, maturation of the neurons cultured in the FCE medium was characterized.

### MATERIALS AND METHODS

#### Materials

Gellan gum (FP001)-containing Neurobasal medium (FCE-M-N) and Dulbecco’s modified Eagle’s medium (FCE-M-D) were prepared at Nissan Chemical Industries (Tokyo, Japan) according to a previously described method. Dulbecco’s modified Eagle’s medium (DMEM) without FP001, poly-L-lysine, anti-microtubule-associated protein 2 (MAP2), anti-βIII-tubulin, anti-Synapsin I, and anti-β-actin antibodies, and anti-mouse and -rabbit immunoglobulin G (IgG) antibodies, conjugated with peroxidase, were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Neurobasal medium without FP001 was purchased from Thermo Fisher Scientific (Malvern, PA, U.S.A.). Anti-vesicular glutamate transporter 1 (VGLUT1) antibody and non-fat dry milk were supplied by Cell Signaling Technology (Danvers, MA, U.S.A.). Fetal bovine serum (FBS) was obtained from Biowest (Nuaille, France). ISOGEN was purchased from Nippon Gene (Tokyo, Japan). MultiScribe™ Reverse Transcriptase was supplied by Applied Biosystems (Foster City, CA, U.S.A.).

THUNDERBIRD SYBR qPCR Mix and Can Get Signal were obtained from TOYOBO (Osaka, Japan). All other chemicals and reagents were of the highest purity available and were purchased from commercial sources.

#### Animals

Pregnant C57BL/6-Tg (CAG-EGFP) mice and ICR mice were purchased from Japan SLC (Hamamatsu, Japan). The mice were housed under pathogen-free conditions under controlled temperature (21–25°C) with a 12:12 light/dark cycle. The lights remained on from 8:00 to 20:00, and food and water were available to the mice ad libitum. The animals were cared for in strict compliance with the guidelines outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal procedures were approved by the Kanazawa University Animal Care Committee.

#### Neuronal Culture

Primary cortical neuronal cultures were performed according to the methods described by Nakamichi et al., with minor modifications. In brief, cerebral cortices from 15-d-old embryonic C57BL/6-Tg (CAG-EGFP) mice or ICR mice were dissected and incubated with 0.25% trypsin in phosphate-buffered saline (PBS) containing 28 mM glucose at 37°C for 20 min. Cells were mechanically dissociated using a 1000-µL pipette tip in Neurobasal medium or DMEM and plated at a density of 7.5 × 10⁵ cells/mL into glass-bottom or plastic dishes that were coated with 7.5 µg/mL poly-L-lysine, for two-dimensional adhesion cultures. The remaining cellular suspension was centrifuged and resuspended in FCE-M-N or FCE-M-D. These cells were plated at a density of 2.25 × 10⁶, 4.50 × 10⁶, and 7.50 × 10⁶ cells/mL into non-coating glass-bottom or plastic dishes, for three-dimensional cultures. Cortical neurons were cultured in Neurobasal medium or FCE-M-N that was supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, B-27 supplement, and 0.5 mM glutamine, or in DMEM or FCE-M-D that was supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 28 mM glucose, 2 mM glutamine, 5 mM N-(2-hydroxyethyl)piperazine-N-2-ethanesulfonic acid (HEPES), 50 µg/mL apo-transferrin, 500 ng/mL insulin, 1 pM β-estradiol, 3 nM triiodothyronine, 20 nM progesterone, 8 ng/mL sodium selenite, and 100 µM putrescine, at 37°C in a humidified atmosphere under 5% CO₂.

#### Three-dimensional Imaging

For two-dimensional cultures, cortical neurons derived from C57BL/6-Tg (CAG-EGFP) mice were seeded at 1.5 × 10⁵ cells/0.2 mL/well into Lab-Tek® chambered cover glass, and cultured for 3 d in Neurobasal medium or DMEM. For three-dimensional cultures, cortical neurons from CAG-EGFP mice were seeded at 4.5 × 10⁵, 9.0 × 10⁵, and 1.5 × 10⁶ cells/0.2 mL/well into Lab-Tek® chambered cover glass, and cultured for 3 d in FCE-M-N or FCE-M-D. These neurons were observed with Z-stack image acquisition using a confocal laser-scanning microscope (LSM710, Carl Zeiss, Jena, Germany) equipped with an argon laser. Fluorescence images were acquired using an excitation wavelength of 488 nm. The parameters of illumination and detection were digitally controlled to ensure that the same settings were maintained throughout the experiments.

#### Quantitative RT-PCR

For two-dimensional cultures, cortical neurons isolated from ICR mice were seeded at 7.5 × 10⁵ cells/mL/well into 12-well plastic dishes, cultured for 3 d in Neurobasal medium or DMEM, and washed with ice-cold PBS. Cells were centrifuged at 4°C for 5 min at 15000 × g after cell harvesting. For three-dimensional cultures, cortical neurons two-dimensionally cultured in the medium without FP001 and the neurons three-dimensionally cultured in the medium with FP001 and the neurons two-dimensionally cultured in the medium without FP001, maturation of the neurons cultured in the FCE medium was characterized.
neurons isolated from ICR mice were seeded at 2.25 × 10^6 cells/mL/well into 12-well plastic dishes, and cultured for 3 d in FCEm-N or FCEm-D. Cultures were centrifuged at 4°C for 5 min at 2000 × g, and supernatant was removed by aspiration. Pellets were washed with ice-cold PBS, followed by centrifugation at 4°C for 5 min at 15000 × g. Total RNA was extracted from pellets thus obtained according to the standard ISOGEN procedure. cDNA was synthesized with oligo (dT)_18 primer, deoxynucleotide triphosphate mix, RT buffer, and MultiScribe™ Reverse Transcriptase, and amplified on a Mx3005P (Agilent Technologies, Santa Clara, CA, U.S.A.) in a reaction mixture containing cDNA with relevant sense and antisense primers (Table 1), and THUNDERBIRD SYBR qPCR Mix. PCR reactions were initiated by template denaturation at 95°C for 15 min, followed by 40 cycles of amplification (denaturation at 95°C for 10 s, and primer annealing and extension at 60°C for 30 s). Relative quantification of expression levels of the target genes was determined by the \( \Delta \Delta C_{\text{T}} \) method using transcripts of acidic ribosomal phosphoprotein P0 (36B4) as the internal standard.

**Western Blot Analysis** Western blot analysis was performed according to the methods described by Nakamichi et al., with minor modifications. For two-dimensional cultures, cortical neurons from ICR mice were seeded at 7.5 × 10^5 cells/mL/well into 12-well plastic dishes, cultured for 3 d in Neurobasal medium, and washed with ice-cold PBS. Cells were centrifuged at 4°C for 5 min at 15000 × g after cell harvesting. For three-dimensional cultures, cortical neurons from ICR mice were seeded at 2.25 × 10^6 cells/mL/well into 12-well plastic dishes, and cultured for 3 d in FCEm-N. Cultures were centrifuged at 4°C for 5 min at 2000 × g, and supernatant FCEm were removed by aspiration. Pellets were washed with ice-cold PBS, followed by centrifugation at 4°C for 5 min at 15000 × g. Cerebral cortices from 15-d-old embryonic ICR mice were dissected, and centrifuged at 4°C for 5 min at 15000 × g after washing with ice-cold PBS. Pellets thus obtained from cultured neurons and cerebral cortices were resuspended and sonicated in 20 mM Tris–HCl buffer (pH 7.5) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylenediaminetetraacetic acid (EGTA), 10 mM sodium fluoride, 10 mM sodium β-glycerophosphate, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 1 µg/mL of various protease inhibitors ([p-aminophenyl]methanesulfonyl fluoride, leupeptin, antipain, and benzamidine). The suspensions were then added at a volume ratio of 4:1 to 10 mM Tris–HCl buffer (pH 6.8) containing 10% glycerol, 2% sodium dodecylsulfate, 0.01% bromophenol blue, and 5% mercaptoethanol, and mixed at room temperature for one hour. The protein concentration was determined using a Protein Assay Kit (BioRad Laboratories, Hercules, CA, U.S.A.). Each aliquot of 10 µg proteins was loaded on to a 7.5% polyacrylamide gel for electrophoresis at a constant current of 21 mA/plate for 30 min at room temperature, using a compact-slab size poly-acrylamide gel electrophoresis (PAGE) system (ATTO, Tokyo, Japan), followed by blotting to a polyvinylidene fluoride membrane that was previously treated with 100% methanol. The membrane was blocked with 5% non-fat dry milk or 2% bovine serum albumin (BSA) solution at 4°C overnight. The membrane was then incubated with antibodies against VGLUT1 (1:5000), that were diluted with buffer containing 5% non-fat dry milk, or against MAP2 (1:10000), Synapsin I (1:50000) or β-actin (1:500000), that were diluted with buffer containing 0.2% BSA, at room temperature for 2 d while shaking. The membrane was then washed and incubated with an anti-mouse IgG1 (1:10000–100000) and anti-rabbit IgG (1:5000–100000), that were conjugated with peroxidase and diluted with 5% non-fat dry milk or 0.2% BSA, at room temperature for 2 d while shaking. Proteins reactive with these antibodies were detected with the aid of ECL Prime™ or ECL™ detection reagents using a lumino image analyzer (LAS-4000, FUJIFILM, Tokyo, Japan). Densitometric determination was performed using ImageJ software.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay** Mitochondrial activity as an index of cell survival was determined by using the MTT assay according to the method described by Nakamichi et al., with minor modifications. For two-dimensional cultures, cortical neurons from ICR mice were seeded at 7.5 × 10^5 cells/mL/well into 24-well plastic dishes, and cultured for 3 d in Neurobasal medium. The cells were exposed to glutamate or hydrogen peroxide for 24 h and washed with ice-cold PBS. For three-dimensional cultures, cortical neurons from ICR mice were seeded at 2.25 × 10^6 cells/mL/well into 24-well plastic dishes, and cultured for 3 d in FCEm-N. The cells were exposed to glutamate or hydrogen peroxide for 24 h and centrifuged at room temperature for 5 min at 2000 × g, and supernatant FCEm were removed by aspiration. Pellets were washed with ice-cold PBS, followed by centrifugation at room temperature for 5 min at 2000 × g. These two- and three-dimensional cultured cells were incubated with MTT solution (0.5 mg/mL in PBS) for 1 h at 37°C. Subsequently, solubilizing solution (0.04 M HCl in isopropanol) at volume equivalent to the MTT solution was added, and the mixture was well shaken for 10 min to dissolve the formazan. The absorbance of the dissolved suspension at 570 nm was measured using a microplate.

### Table 1. Primers Used for Real-Time Polymerase Chain Reaction

| Genes   | Sense primers                                                                 | Antisense primers                   |
|---------|-------------------------------------------------------------------------------|-------------------------------------|
| mPNMT   | AGACCTGACCAACCCCTGATG                                                         | CCGGAGCACAATATCAATGAG               |
| mTH     | GCAGCCCTACCAAGATCAAAA                                                         | CAGGGTGTAAGGGTCAACT                 |
| mVGLUT1 | TCGTGGCTCGAGTGCACCC                                                         | TGGTAAGCCCAAGAAGAGAG                |
| mGAD1   | ATACAACCTTTGGCTGATGT                                                       | GAGGGTTCCAGTGGTACTGA                |
| mChAT   | CTCGCCACGGCTCTTCTGAGT                                                       | GAGTGCCTACACACACACACAC              |
| mTPH    | GGGCTGGTGAAAGGACCTATTG                                                      | ATGCGACCCGACGAGATT                  |
| mDjH    | TTTGTGGTGACCGGTACCTG                                                        | GGAGGACAGTAAACCACCTT                |
| m36B4   | ACTGGTCTAGGACCCGAGAAG                                                       | TCCACCTGTGTCACAGTCT                 |
Statistical Analysis  All experiments were performed at least three times. Data were expressed as the mean ± standard error of the mean (S.E.M.) The statistical significance of differences was determined by means of Student’s t-test or one-way ANOVA with the Tukey–Kramer test, and \( p < 0.05 \) denoted a statistically significant difference.

RESULTS

Morphology of Three-Dimensionally Cultured Neurons in the FCeM  We have first investigated whether neurons can three-dimensionally extend neurites in the FCeM medium. When neurons derived from cortices of C57BL/6-Tg (CAG-EGFP) mice were seeded at the same cell density and cultured for 3d, the density of the cells in FCeM-N (A–D) or FCeM-D (E–H) were cultured in the FCeM-N (A–D) or FCeM-D (E–H). Panel (B) and (F) show high-magnification observation of panel (A) and (E), respectively. The experiment was repeated in triplicates, and typical fluorescence images are shown. (Color figure can be accessed in the online version.)

![Fig. 1. Morphology of Mouse Cortical Neurons Cultured in Either Neurobasal Medium with FP001 or DMEM with FP001](image)
in FCEM-N (Figs. 1A, B) was higher than that in FCEM-D (Figs. 1E, F).

Next, distributions of three-dimensionally cultured neurons in the medium containing FP001 was compared with those two-dimensionally cultured in the medium not containing FP001. Neurons cultured in the Neurobasal medium (Fig. 2A) or DMEM (Fig. 2C) at 7.5 × 10⁵ cells/mL were two-dimensionally distributed on the bottom of the culture dish.
in the plane direction. On the other hand, neurons cultured in the FP001-containing medium were three-dimensionally distributed not only on the bottom of the dish, but also throughout the media within the observation range. Thus, it was shown that three-dimensional extension of neurites can be observed for neurons cultured in the FP001-containing medium.

**Expression of Neurotransmitter Phenotype-Related Genes in the Cultured Neurons**

Neurotransmitter phenotype was next examined in the neurons three-dimensionally cultured in the FP001-containing medium. In neurons cultured in FCeM-N and FCeM-D, mRNA expression of the glutamatergic neuronal marker, VGLUT1, and the dopaminergic neuronal marker, tyrosine hydroxylase (TH), was higher and tended to be higher, respectively, whereas that of the GABAergic neuronal marker, glutamate decarboxylase (GAD), was lower, compared to that in neurons cultured in the Neurobasal medium and DMEM without FP001, respectively (Figs. 3A, B). Since these observations were common in the two media, it was speculated that three-dimensional culture using FP001 would be responsible for the difference in neurotransmitter phenotype. mRNA expression of the adrenergic neuronal marker, phenylethanolamine N-methyltransferase (PNMT), was detected in neurons cultured in the Neurobasal medium, but not in DMEM. The expression of PNMT was higher in cells grown in the FCeM-N compared to the Neurobasal medium (Fig. 3A). In addition, protein expression of VGLUT1 in neurons cultured in the FCeM-N and in cerebral cortex of embryonic mice tended to be higher than those cultured in the Neurobasal medium (Fig. 3C). These results suggested that three-dimensional culture using FP001 may promote maturation into excitatory neurons and suppress maturation into inhibitory neurons.

**Effects of the FCeM Medium on Neuronal Maturation in Cultured Neurons**

Three-dimensional culture using FP001 accelerated the differentiation of HepaRG cells into mature hepatocytes, suggesting that primary culture using FP001 may promote cellular maturation. Therefore, neuronal maturation was next examined in neurons three-dimensionally cultured in the FCeM-N. Expression of the mature neuronal marker, MAP2a,b, and the synapse marker, Synapsin I, in neurons cultured in the FCeM-N was significantly higher than that in neurons cultured in the Neurobasal medium without FP001 (Fig. 4). Expression of MAP2a,b in neurons cultured in the FCeM-N was more similar to that in cerebral cortex of embryonic mice, compared to neurons cultured in the Neurobasal medium (Fig. 4). These results suggested that three-dimensional culture using FP001 may promote neuronal maturation, and neurons in the FP001-containing medium could be cultured in the conditions similar to those in embryonic brain.

**Effects of the FCeM on Sensitivity to Glutamate in Cultured Neurons**

Since the data of mRNA (Figs. 3A, B) and protein expression (Fig. 3C) may suggest that glutamatergic neurons may be increased by culturing neurons in the FCeM, we next investigated whether culturing in the FCeM affects the sensitivity of neurons to glutamate. Exposure to 100–1000 µM glutamate for 24 h minimally changed MTT reduction in neurons cultured in the Neurobasal medium (Fig. 5A). On the other hand, exposure to 300 µM or higher concentration of glutamate significantly enhanced MTT reduction in neurons cultured in the Neurobasal medium (Fig. 5A). On the other hand, exposure to 100 µM H2O2 for 24 h significantly suppressed MTT reduction regardless of the presence of FP001 (Fig. 5B). These results suggested that three-dimensional culture using FP001 may increase mitochondrial activity in response to glutamate. On the other hand, exposure to 100 µM H2O2 for 24 h significantly suppressed MTT reduction regardless of the presence of FP001 (Fig. 5), indicating that both of the cultured neurons are responsive to oxidative stress induced by H2O2.
DISCUSSION

The present study demonstrated that neurons can be three-dimensionally cultured in the FCeM (Figs. 1, 2). There have been many studies that have reported using embedding culture method for conventional three-dimensional neuronal culture. In embedding culture, however, gels or fibers, used as scaffolds, may obstruct the diffusion of compounds added into the medium and the collection of cells aside from gels or fibers. This may provide difficulty in evaluating the activity of compounds in the neurons. On the other hand, the viscosity of the FCeM is almost equal to that of water, and the compounds added can easily diffuse in the medium. Furthermore, since neurons can be easily separated from FP001 by centrifugation, the cells can be easily isolated. In fact, in the present study, we evaluated the effect of glutamate, added into the medium, on MTT reduction ability of neurons (Fig. 5). Expression of various neuronal markers in neurons cultured in the FCeM was also examined by quantitative PCR and Western blotting (Figs. 3, 4). Thus, by mixing the functional polymer, FP001, into the culture medium, a novel three-dimensional culture system for neurons facilitating easier drug addition and cell collection was constructed.

In vitro models of neurodegenerative disorders have previously been produced by the addition of neurotoxins into the medium. FP001 may be used as a scaffold to produce such in vitro pathological models using three-dimensional cultured neurons. Furthermore, in vitro neurodegenerative disorder model using FP001 may be applied to the assay of neuroprotective effects induced by the addition of drugs.

The expression of the matured neuronal marker, MAP2a,b, and the synapse formation marker, Synapsin I, in neurons three-dimensionally cultured in the FCeM was significantly higher than that in neurons two-dimensionally cultured in FP001-free medium (Fig. 4), suggesting that culture using FP001 may promote neuronal maturation and synapse formation. Neurons extend neurites, connect to other neurons, and form synapses. The synapse formation is one of the key processes in the formation of neuronal networks. In three-dimensionally cultured neurons, neurites are extended in the vertical direction along with the planar direction (Fig. 1), and neurons may interact with other neurons not only in a plane, but also in a stereoscopic manner. Thereby, synapse formation may be promoted in three-dimensionally cultured neurons. It might be also noteworthy that the expression pattern of MAP2a,b in three-dimensionally cultured neurons was more similar to that in the mouse embryonic cerebral cortex, compared to neurons two-dimensionally cultured in the FP001-free medium (Fig. 4). Certain clinically used drugs, such as selective serotonin reuptake inhibitors and β2 stimulants, exhibit neurotoxicity in the embryonic brain as adverse effects. The three-dimensional neuronal culture using FP001 may be employed for the evaluation of neurotoxicity in the embryonic brain.

Glutamate is a major excitatory neurotransmitter that regulates brain functions, such as learning and memory, in the central nervous system. Excessive activation of glutamate receptor due to elevation of extracellular glutamate concentration causes excitotoxicity in neurons and is involved in the onset and/or development of neurodegenerative diseases including Alzheimer’s and Parkinson’s diseases. The mRNA expression of the glutamatergic neuronal marker, VGLUT1, in neurons three-dimensionally cultured in FP001-containing medium was higher than that in two-dimensionally cultured neurons without FP001 (Fig. 3), suggesting that neuronal culture using FP001 may promote neurotransmission of glutamatergic neurons. Indeed, MTT reduction activity was minimally altered by the addition of glutamate into the medium in two-dimensionally cultured neurons without FP001 (Fig. 5A), but
was significantly enhanced after the exposure to 300–1000 µM glutamate in neurons three-dimensionally cultured with FP001 (Fig. 5B). Under the present experimental conditions, neurotoxicity seemed to not be induced by exposure to high concentration of glutamate (Fig. 5). Glutamate neurotoxicity is triggered by excessive influx ofCa^{2+} into intracellular space through N-methyl-D-aspartate (NMDA) receptor channels^{34,35} and usually evoked at the concentrations of 50–100 µM glutamate.^{36,37} On the other hand, glutamate does not induce excitotoxicity in immature neurons.^{36–38} Such difference could be due to the differences in the NMDA receptor subunits expressed between mature and immature neurons.^{39,40} Since immature neurons cultured for 3 d were used in the present study, excitotoxicity by glutamate might not be induced (Fig. 5). In the early stages of brain development, glutamate is thought to be neurotrophically active,^{41,42} and may act as a neurotrophin in neurons cultured with FP001, which may abundantly include glutamatergic neurons (Fig. 5B). This result also suggested that in vitro experimental system similar to the embryonic brain may be constructed by three-dimensional culture using FP001, compared to two-dimensional one. Since abnormalities in the formation of neuronal networks in the embryonic period are involved in the onset of various neuropsychiatric disorders,^{43} three-dimensionally cultured neurons using FP001 are expected to become an effective analytical tool for elucidation of the onset mechanisms of neuropsychiatric disorders.

Although expression pattern of the mature neuronal marker, MAP2a,b, in neurons that are three-dimensionally cultured may be similar to that in the embryonic brain neurons (Fig. 4), it would be an important research topic in the future to investigate whether the properties of the neurons cultured in the FCEm for a long period are similar to those of the adult brain neurons. Nevertheless, it would be difficult to change the medium without disrupting the three-dimensionally formed neuronal networks, because the viscosity of the FCEm is almost equal to that of water,^{18,19} and no gelation was found in the medium containing FCEm during the culture period in the present study (data not shown). Such problem in the medium change may be solved by constructing the system in another chamber separated by a membrane, which permeates water-soluble solutes in the medium, next to the chamber of neurons three-dimensionally cultured, and the second chamber would be used for supplying necessary substance and/or removing unnecessary medium. Furthermore, to mimic the environment of the living brain, it would be preferable to co-culture the neurons with other surrounding cells, such as astrocytes and microglia. In fact, co-culture of neurons with glial cells promotes formation of mature functional synapse.^{43–45} Thus, the present study provided the first step for three-dimensional primary culture of neurons showing neuronal maturation in presence of FP001, and further studies are required to construct a three-dimensional culture model resembling the adult brain.

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Conflict of Interest The authors declare no conflict of interest. Yukio Kato serves as a consultant to Nissan Chemical Industries, Ltd.

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