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# Improvement in protocol to generate homogeneous glutamatergic neurons from mouse embryonic stem cells reduced apoptosis

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ABSTRACT

Obtaining a homogenous population of central nervous system neurons has been a significant challenge in neuroscience research; however, a recent study established a retinoic acid-treated embryoid bodies-based differentiation protocol that permits the effective generation of highly homogeneous glutamatergic cortical pyramidal neurons from embryonic stem cells. We were able to reproduce this protocol regarding the purity of glutamatergic neurons, but these neurons were not sufficiently healthy for long-term observation under the same conditions that were originally described. Here, we achieved a substantial improvement in cell survival by applying a simple technique: We changed the medium for glutamatergic neurons from the original complete medium to commercially available SBM (the Nerve-Cell Culture Medium manufactured by Sumitomo Bakelite Co. Ltd.) and finally succeeded in maintaining healthy neurons for at least 3 weeks without decreasing their purity. Because SBM contains glial conditioned medium, we postulated that brain-derived neurotrophic factor or basic fibroblast growth factor is the key components responsible for pro-survival effect of SBM on neurons, and examined their effects by adding them to CM. As a result, neither of them had pro-survival effect on pure glutamatergic neuronal population.



## 1. Introduction

A significant problem in neuroscience had been the lack of relevant culture system that allows unlimited generation of defined populations of post-mitotic, process-bearing central nervous system (CNS) neurons. Embryonic stem (ES) cells [1,2] have created the possibility of generating infinite numbers of any types of CNS neurons, and several protocols have been developed for differentiation of ES cells into neurons [3,4]. However, neuronal cultures derived from ES cells contain a variety of neuronal subtypes as well as neural precursor cells (NPCs) and non-neural cells, including glial cells.

Recent advances in culture techniques have overcome this issue. Bibel and co-workers revealed that their retinoic acid-treated embryoid bodies based protocol permits generation of a homogeneous population of glutamatergic cortical pyramidal neurons from ES cells [5,6]. The purity of this population reaches 90–95%, which is the highest for differentiation of glutamatergic neurons ever reported [7,8]. In our hands, this protocol was highly reproducible regarding the purity of ES cell-derived glutamatergic neurons, but these neurons did not survive longer than 2 weeks under the same conditions that were originally described [6]. In addition, we found that low-density culture of neurons resulted in low survival rate, but in order to assess axonal properties, synaptic transmission, and dendritic arborization of glutamatergic neurons, long-term low-density culture is required. Therefore, it is necessary to improve survival of these ES-derived neurons.

Here, we made a substantial improvement in cell survival by using a simple technique: We changed the medium for glutamatergic neurons from the original medium (complete medium, CM) to a commercially available medium (the Nerve-Cell Culture Medium from Sumitomo Bakelite Co. Ltd., SBM) and finally succeeded in

*Abbreviations:* CNS, central nervous system; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; ES cell, embryonic stem cell; NPC, neural progenitor cell; CA, cellular aggregate; $N_2M$, $N_2$ medium; CM, complete medium; SBM, the Nerve-Cell Culture Medium from Sumitomo Bakelite Co. Ltd.; ICC, immunocytochemistry; Tuj1, neuronal class III β-tubulin; VGLUT1, vesicular glutamate transporter 1; DAPI, 4′,6-diamidino-2-phenylinodole; PBS, phosphate-buffered saline.

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keeping neurons healthy for at least 3 weeks without decreasing their purity below 98%.

## 2. Materials and methods

### 2.1. Antibodies and reagents

The following reagents were used for culture of glutamatergic neurons: the Nerve-Cell Culture Medium (SBM, Sumitomo Bakelite Co. Ltd., Tokyo, Japan), recombinant human brain-derived neurotrophic factor (BDNF, Pepro Tech Inc., Rocky Hill, NJ, USA), and recombinant human basic fibroblast growth factor (bFGF, BD Bioscience, Bedford, MA, USA). All other reagents have been described previously [5,6]. For immunocytochemistry, the following antibodies were used: mouse monoclonal antibody to neuronal class III β-tubulin (Tuj1, 1:1000; Covance Laboratories, Inc., Berkeley, CA, USA); rabbit polyclonal antibodies to vesicular glutamate transporter 1 (VGLUT1, 1:1000, Synaptic System, Goettingen, Germany), and cleaved caspase-3 (1:200, Cell Signaling Technology, USA). Fluorescent Mounting Medium was purchased from DakoCytomation Inc., (USA). We used the following fluoroscence-conjugated secondary antibodies: Alexa Fluor 488- or 568-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (1:400, Invitrogen, USA).

### 2.2. Cell culture

The ES cell line we selected was E14TG2a (CRL-1821, American Type Culture Collection, Manassas, VA, USA). ES-cell derived glutamatergic neurons were differentiated as previously described [5,6], until the stage where NPCs differentiated into neurons. We adjusted the $CO_2$ content in the incubators to maintain the pH of each culture medium at about 7.4, which made cells a little healthier. Specifically, we used the following conditions: ES cells in ES medium, cellular aggregates (CAs) in CA medium, neurons in complete medium (CM) with 10% $CO_2$, NPCs in $N_2$ medium ($N_2$M) with 7% $CO_2$, and neurons in SBM with 5% $CO_2$. The temperature of the incubators was kept constant at 37 °C.

We always once froze all of dissociated CAs, which were NPCs, and started all of our experiments from NPCs by thawing and plating them as originally described [5,6]. We plated the NPCs on glass coverslips in 24-well plates at a density of $0.45 \times 10^6$ cells/well in all experiments, except to examine the purity of glutamatergic neurons, where we plated them at a density of $0.9 \times 10^6$ cells/well. Glass coverslips were double coated with poly-dl-ornithine and laminin before use as originally described [6]. After culturing of NPCs in $N_2$M for 2 days, we changed the medium to either CM, as used originally, or to SBM. The time schedule for treating cells either with CM or SBM and fixing treated cells for immunocytochemistry (ICC) is described in Fig. 1a. Because SBM consists of glial conditioned medium and might contain BDNF and bFGF, and CM does not, we hypothesized that these factors were responsible for the improved growth of cells in SBM. To test this hypothesis, we added BDNF and bFGF to CM and measured the effect on longevity and purity. The time schedule for treating cells with BDNF or bFGF and fixing the cells for ICC is described in Figs. 2a and 3a. We tested most of our cells for the presence of mycoplasma as originally recommended [6].

### 2.3. Immunocytochemistry and nuclear staining

Cells cultured on glass coverslips in 24-well plates were washed with phosphate-buffered saline (PBS). After transferring coverslips in new 24-well plates, we fixed cells with 4% paraformaldehyde in 0.1 M phosphate buffer (Wako Pure Chemical Industries, Japan) for 10 min. Cells were washed with PBS and incubated for 10 min in permeabilizing solution (PBS containing 0.2% Triton X). After three washes with PBS, cells were incubated for 1 h in blocking solution (PBS containing 5% bovine serum albumin and 0.05% Tween). Subsequently, cells were incubated with primary antibodies for 1 h at room temperature or overnight at 4 °C. After three washes with PBS, cells were incubated with fluorescence-conjugated secondary antibodies for 1 h at room temperature or overnight at 4 °C. After three washes with PBS, nuclei were counterstained with Hoechst 33342 (Invitrogen) or 4′,6-diamidino-2-phenylinodole (DAPI, Dojindo, Japan). Coverslips were then rinsed three times with PBS and mounted on glass slides. The samples were viewed under an inverted light microscope equipped with epifluorescence and dry condenser for phase-contrast microscopy (DP70, Olympus, Tokyo, Japan) using a 10× objective.

### 2.4. Detection of apoptosis

Hallmarks of apoptotic cell death include activation (cleavage) of caspases, condensation and fragmentation of nuclei, and formation of apoptotic bodies. We investigated caspase-3 activation by using ICC and examined extensive chromatin condensation and nuclear fragmentation using Hoechst staining. Neurons were analyzed for immunofluorescent labeling for neuronal marker Tuj1 (green) and for apoptotic marker cleaved caspase-3 (red), and nuclei were counterstained with Hoechst 33342 or DAPI (blue). We obtained 4 representative images per well randomly under the microscope with a 10× objective, and counted all of the cells in those images for one experiment. We quantified the percentage of cleaved caspase-3-positive neurons (cleaved capase-$3^+$ cells per all Tuj$1^+$ neurons) in each culture. More than 1500 cells were counted in each of three separate independent experiments to quantify cleaved caspase-3-positive cells.

### 2.5. Statistical analysis

The quantitative data are expressed as mean ± SEM of three independent experiments. Statistical analysis of these values was performed using Student's $t$ test. Values of $P < 0.05$ were considered statistically significant.

## 3. Results

### 3.1. SBM remarkably prolonged the survival of ES cell-derived glutamatergic neurons without decreasing their purity

Bibel and co-workers recently established a differentiation protocol that generates highly homogeneous glutamatergic cortical neurons from ES cells [5,6]. This protocol was highly reproducible regarding the purity of ES cell-derived glutamatergic neurons in our hands, but these neurons did not survive longer than 2 weeks under the same conditions that were originally described [6]. We quantified apoptotic neurons by investigating caspase-3 activation by using ICC and found that 60% of the neurons underwent apoptosis within 6 d after plating (Fig. 1b, left). These apoptotic cleaved caspase-3-positive neurons also showed other hallmarks of apoptosis, including extensive chromatin condensation and nuclear fragmentation, as assessed by Hoechst staining (data not shown). In order to improve neuronal survival, we modified this protocol by changing the medium for glutamatergic neurons from CM to SBM 48 h after plating (SBM protocol, Fig. 1a). We continued to culture neurons in CM or SBM for 4 d and then examined caspase-3 activation by using ICC. SBM significantly decreased the percentage of cleaved caspase-3-positive cells from 60% to 20% (Fig. 1b and c); in addition, we verified that neurons remained healthy for at least

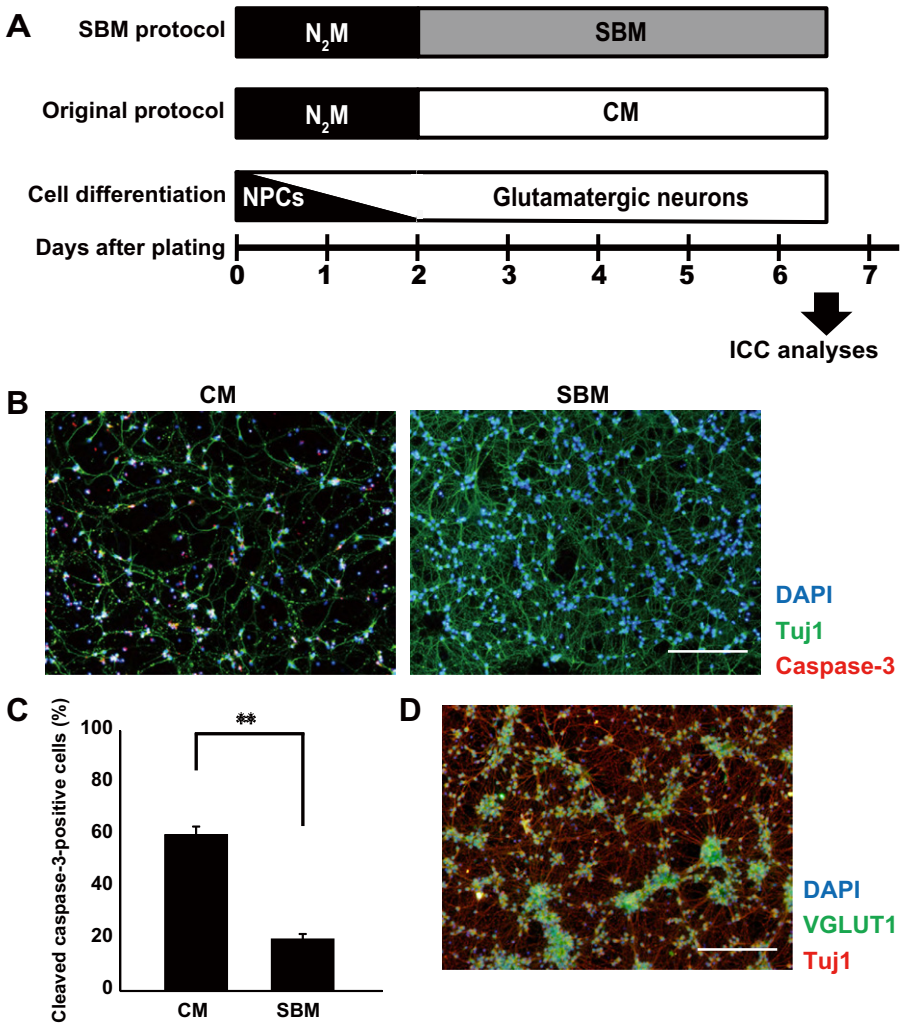


**Fig. 1.** SBM remarkably prolonged the survival of ES cell-derived glutamatergic neurons without decreasing their purity. Glutamatergic neurons were cultured in CM (original protocol) or SBM (modified protocol) from 48 h after plating, followed by investigation of caspase-3 activation by using ICC 4 d later. (A) Schematic illustration of SBM protocol. Scheme shows the part that we modified in the original protocol and how NPCs differentiate into glutamatergic neurons. (B) Representative images of neurons 6 d after plating, cultured in CM (left) or SBM (right). Neurons were analyzed for immunofluorescent labeling with the neuronal marker Tuj1 (green) and apoptosis marker cleaved caspase-3 (red). Nuclei were counterstained with DAPI (blue). Scale bar: 200 μm. (C) Quantification of the percentage of cleaved caspase-3-positive neurons (cleaved capase-3$^+$ cells per all Tuj1$^+$ neurons) 6 d after plating, cultured in CM or SBM. We obtained four representative images, and counted all of the cells in those images (more than 1500 cells) for one experiment. Note that SBM significantly decreased the percentage of cleaved caspase-3-positive cells from 60% to 20%. Values represent the mean ± SEM of three separate experiments. $^{**}P < 0.05$ by Student's *t* test. (D) A representative image of neurons 7 d after plating, cultured in SBM. Note that over 98% neurons are VGLUT1-positive. Neurons were analyzed for Tuj1 (red) and for glutamatergic neuron marker VGLUT1 (green). Nuclei were counterstained with DAPI (blue). Scale bar: 200 μm.

3 weeks. SBM contains glial conditioned medium, which might increase the number of non-neural cells. To confirm the purity of cultures grown in SBM, we measured the percentage of cells positive for the glutamatergic neuronal marker VGLUT1 by using ICC 7 d after plating. The purity of our ES cell-derived glutamatergic neurons was more than 98%, higher even than the purity of cultures grown in CM (90–95%) [5,6] (Fig. 1d).

### *3.2. Neither BDNF nor bFGF are responsible for the pro-survival effect of SBM on ES-glutamatergic neurons*

SBM consists of glial conditioned medium which might contain both BDNF and bFGF. We postulated that BDNF, which is a potent neurotrophic factor secreted from astrocytes, is responsible for the pro-survival effect of SBM on glutamatergic neurons [9,10]. To test this hypothesis, we added BDNF (1, 10, 50, or 100 ng/ml) in $N_2M$ and CM just after plating and investigated caspase-3 activation 7 d later (Fig. 2a, BDNF protocol-0 h). Compared to the control, BDNF demonstrated no pro-survival effect on neurons (Fig. 2b, upper panels). To optimize the timing of BDNF treatment, we added 50 ng/ml BDNF in $N_2M$ and CM or only in CM 24 or 48 h after plating, respectively, and investigated caspase-3 activation 7 d after plating (Fig. 2a, BDNF protocol −24 and −48 h). The BDNF protocol again had no pro-survival effect on neurons (Fig. 2b, middle and lower panels).

In order to enhance the survival of NPCs and neurons, we also added bFGF (1.3, 13.3, 133.3 ng/ml, or 1.3 μg/ml) [11–13] in $N_2M$ and CM just after plating and investigated caspase-3 activation 6 d later (Fig. 3a, bFGF protocol-0 h). Compared to the control, despite remarkable dose-dependent induction of proliferation of non-neuronal glial-like cells, bFGF showed no pro-survival effect on neurons (Fig. 3b). In our culture system, almost all cells are NPCs of glutamatergic neurons just after thawing and plating and they are designed to differentiate into glutamatergic neurons within less than 48 h (Fig. 1a). To reduce proliferation of non-neuronal glial-like cells, we waited for 48 h for programmed differentiation of NPCs into neurons, and then, we added bFGF (2, 10, 100 ng/ml, or 1 μg/ml) in CM 48 h after plating and investigated caspase-3

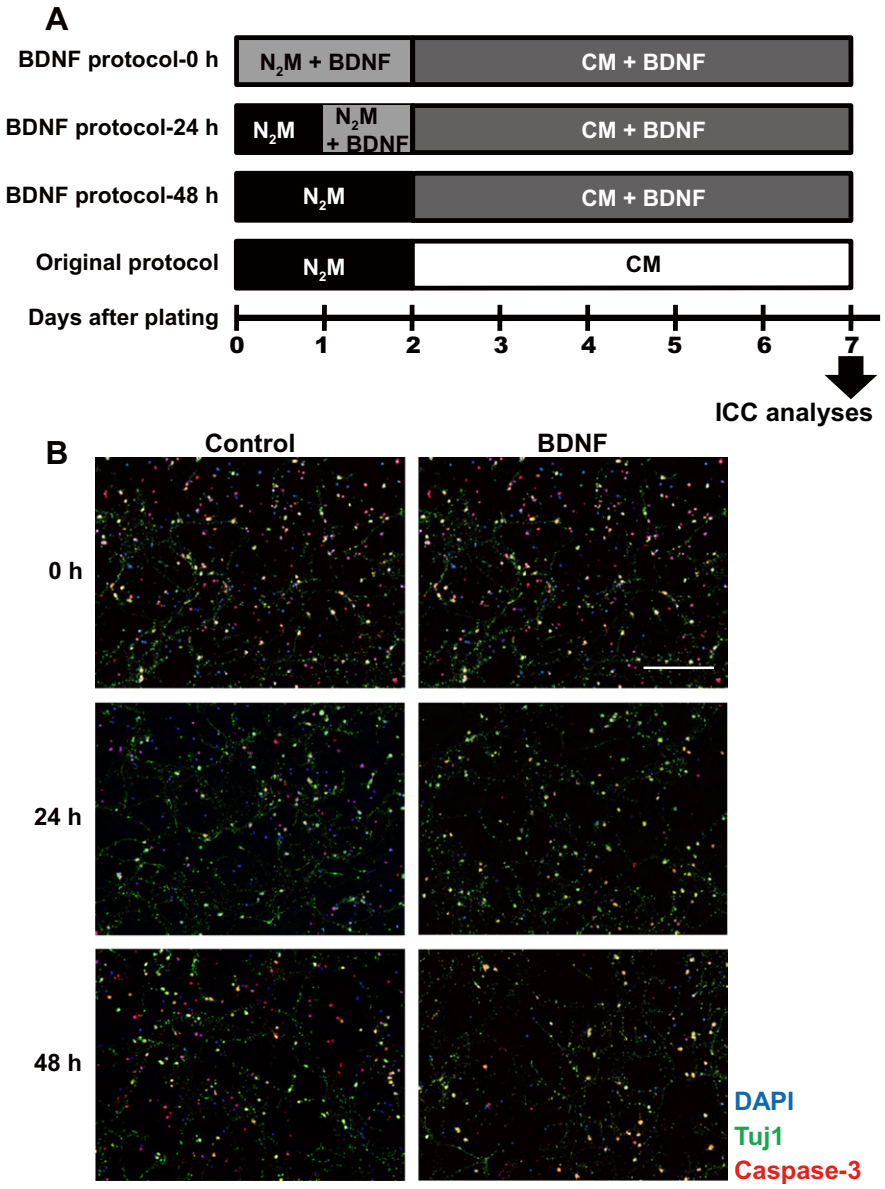


**Fig. 2.** BDNF showed no pro-survival effect on ES cell-derived glutamatergic neurons regardless of timing of addition to the medium. Glutamatergic neurons were cultured in $N_2M$ and CM, which were added BDNF (1, 10, 50, or 100 ng/ml) just after plating, followed by investigation of caspase-3 activation by using ICC 7 d later. Cells were also cultured in $N_2M$ and CM or only in CM, which were or was added 50 ng/ml BDNF 24 or 48 h after plating, respectively, followed by investigation of caspase-3 activation by using ICC 7 d after plating. (A) Schematic illustration of BDNF protocols. Scheme shows how we added BDNF into the medium. (B) Representative images of neurons 7 d after plating, treated with control (left) or BDNF (right). Cells were treated at 0 (upper panels), 24 (middle panels), or 48 h (lower panels) after plating. The representative image of neurons treated with 100 ng/ml BDNF is shown as that of BDNF protocol-0 h. Note that BDNF showed no pro-survival effects on neurons. Neurons were analyzed for immunofluorescent labeling of Tuj1 (green) and cleaved caspase-3 (red). Nuclei were counterstained with DAPI (blue). Scale bar: 200 μm.

activation 5 d later (Fig. 3a, bFGF protocol-48 h). Compared to the control, although induction of proliferation of non-neuronal glial-like cells was remarkably down-regulated, bFGF showed no pro-survival effect on neurons (Fig. 3c).

These results indicate that the main player in the pro-survival effect of SBM on ES-glutamatergic neurons was neither BDNF nor bFGF.

## 4. Discussion

Bibel et al. recently established a sophisticated protocol in which a homogeneous population of glutamatergic cortical pyramidal neurons can be obtained by inducing differentiation from ES cells [5,6], but these neurons did not survive longer than 2 weeks under the original condition unlike regular mixed cortical neuronal culture. In the present study, we made a substantial improvement in the original protocol. Our study revealed that changing the medium for glutamatergic neurons from the originally designated CM to commercially available SBM could robustly prolong the survival of glutamatergic neurons without decreasing their purity. Although we postulated BDNF [9,10] and bFGF [11–13] as the main players of the pro-survival effect of SBM on neurons, neither factor generated a pro-survival effect on neurons when it was added to CM, regardless of the timing. It is possible that a combination of BDNF and bFGF could have an additive effect [14], but the fact that each one on its own did not alter survival rate suggests that neither of them is a main player.

Considering the short survival of neurons in our system, we performed some recommended troubleshooting, including testing all of our cells for mycoplasma infection, which can influence

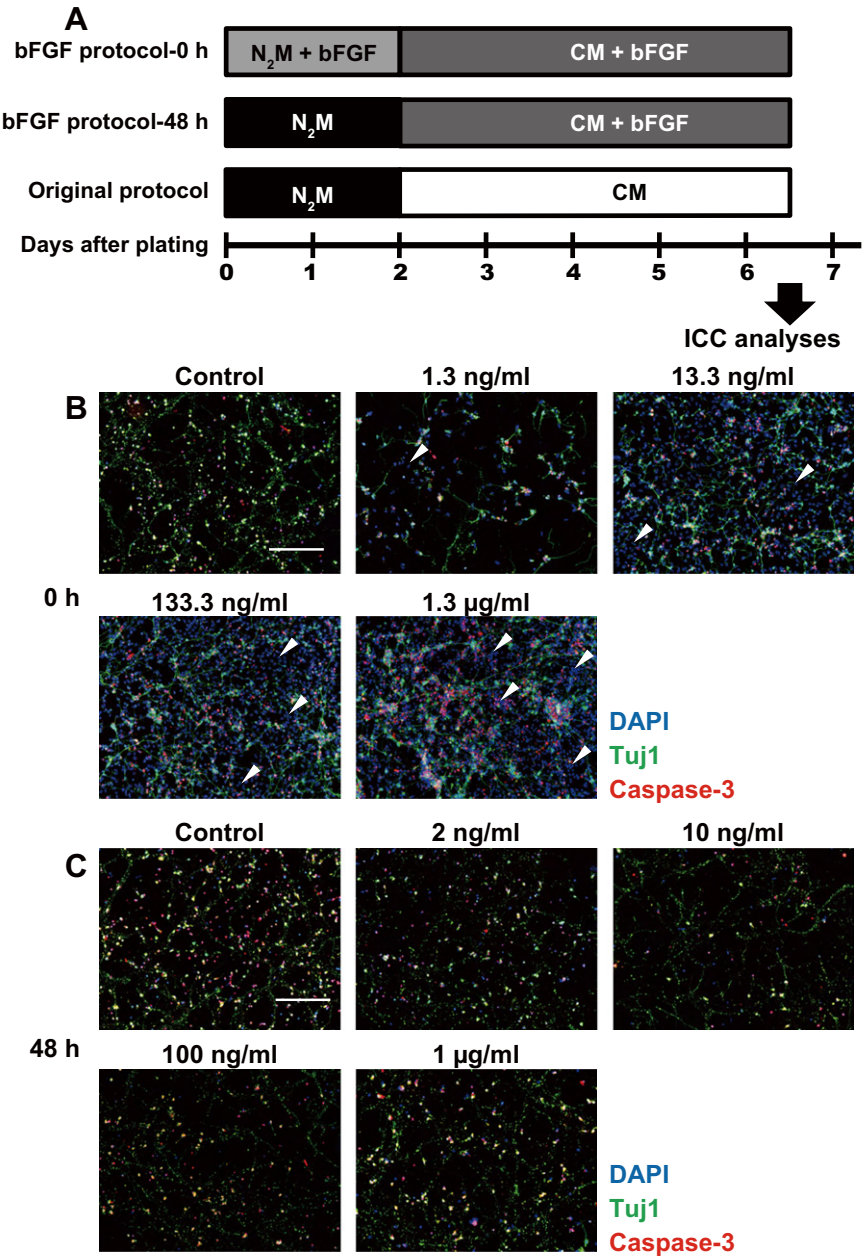


**Fig. 3.** bFGF showed no pro-survival effect on ES cell-derived glutamatergic neurons regardless of timing of addition to the medium. Glutamatergic neurons were cultured in $N_2M$ and CM, which were added bFGF (1.3, 13.3, 133.3 ng/ml, or 1.3 μg/ml) just after plating, followed by investigation of caspase-3 activation by using ICC 6 d later. Cells were also cultured in CM which was added bFGF (2, 10, 100 ng/ml, or 1 μg/ml) 48 h after plating, followed by investigation of caspase-3 activation by using ICC 5 d later. (A) Schematic illustration of bFGF protocols. Scheme shows how we added bFGF into the medium. (B) Representative images of neurons treated with control or bFGF for 6 d from just after plating. Despite remarkable dose-dependent induction of proliferation of non-neuronal glial-like cells, bFGF showed no pro-survival effect on neurons. (C) Representative images of neurons treated with control or bFGF for 5 d from 48 h after plating. Although induction of proliferation of non-neuronal glial-like cells was remarkably low, bFGF showed no pro-survival effect on neurons. In (B) and (C), neurons were analyzed for immunofluorescent labeling of Tuj1 (green) and cleaved caspase-3 (red). Nuclei were counterstained with DAPI (blue). Non-neuronal cells are $DAPI^+$ $Tuj1^-$ glial-like cells indicated by white arrowheads. Scale bar: 200 μm.

differentiation [6]. We also made several minor modifications that were not originally described but were better for neuronal survival, such as altering the $CO_2$ content in the incubators to maintain the pH at 7.4. Another possible issue might be the difference in genetic background between our ES cell line, E14TG2, and the cells lines used in the original protocol [6]; however, E14TG2 is one of the established ES cell lines for general usage. Therefore, we conclude that homogeneity of neurons could be a biological cause of glutamatergic neuronal death.

Neurotrophic factors are released from several possible sources, including target tissues, neuronal networks, neurons themselves (autocrine), glial cells, blood vessels, and other organs within the hormonal network. Our results show that neurotrophic factors from glia are the most powerful pro-survival factors for homogenous populations of glutamatergic neurons and neither BDNF nor FGF showed a pro-survival effect against them. Another possible cause of death is that toxic factors released from the glutamatergic neurons themselves such as glutamate actually kill the cells. Regarding regular cortical neuronal culture, which is established without astrocytes, mixed neuronal culture with heterogeneous neurons also seems to facilitate glutamatergic neuronal survival. Based on these results, regardless of the source of the neurotrophic factors, a homogenous population of glutamatergic neurons without trophic support undergoes spontaneous death.

What is the major neurotrophic factor for glutamatergic neurons? There might be many candidates other than BDNF and bFGF. Astrocytes release a variety of trophic factors both under normal conditions and after brain injury, and these are likely to influence neuronal survival and plasticity [9,15–18]. These trophic factors include, in addition to BDNF and bFGF, nerve growth factor (NGF) [19–21], leukemia inhibitory factor (LIF) [22], interleukin-6 (IL-6) [23,24], ciliary neurotrophic factor (CNTF) [18,19,25], vascular endothelial growth factor (VEGF) [16,26], glial cell-derived neurotrophic factor (GDNF) [27–29], insulin growth factor-1 (IGF-1) [30,31], and others [16,17], and some of their pro-survival effects are reported to be as powerful as that of BDNF and bFGF.

Although further studies are required to clarify the main player involved in the pro-survival effect of SBM on ES-derived glutamatergic neurons, this is the first report demonstrating a substantial improvement in the original protocol, allowing the generation of homogeneous glutamatergic neurons from mouse ES cells.

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