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A simple DMSO-based method for cryopreservation of primary hippocampal and cortical neurons

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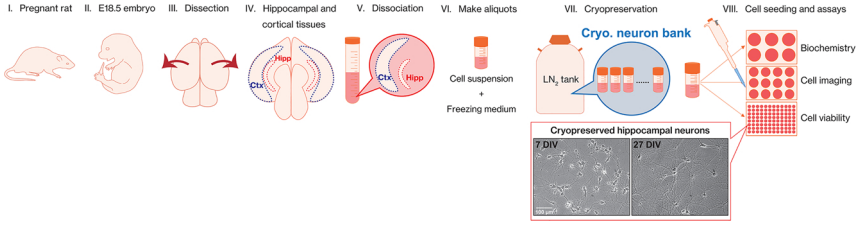
GRAPHICAL ABSTRACT



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ABSTRACT

Background: Primary neuronal cultures are widely used to elucidate fundamental aspects of neuronal anatomy, physiology, cell biology, and neuronal dysfunction in animal models of disease. However, preparation of primary neuronal cultures from rodent embryos is labor-intensive, and it is often difficult to produce high-quality cultures consistently in a single laboratory, and to compare results between laboratories. To overcome these issues, cryopreservation can be used to obtain more standardized, high-quality banks of neuronal cultures.
New Method: In this study, we present a simplified cryopreservation method for rodent primary hippocampal and cortical neurons from embryonic day 18.5 fetuses, using DMSO-containing traditional cell freezing medium.
Results: Cryopreserved neurons stored for more than 1 year in liquid nitrogen were assessed by cell imaging, as well as biochemical signaling transduction and gene expression in response to pharmacological treatments. Cryopreserved neuronal cultures were comparable to freshly prepared cultures in terms of: (1) neuronal viability, (2) neuronal morphology and maturation, (3) functional synapse formation, (4) stimulus responsiveness. These results indicate that DMSO-cryopreserved neurons are equivalent to freshly prepared neurons both developmentally and functionally.
Comparison with Existing Methods: Our method is simple and does not require special reagents or equipment.
Conclusions: Introduction of the cryopreserved neurons as a standard laboratory practice has the potential to increase the robustness and reproducibility of findings between laboratories and reduce the number of animals used in research.

1. Introduction

Primary neuronal cultures are commonly used to study cellular and molecular processes in detail. However, there are several limitations for making primary neuronal cultures in comparison with immortalized cell lines. Preparation of neuronal cultures is labor-intensive and the dissection of the hippocampus and other small brain structures from embryos and neonates is technically challenging. In addition,

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appropriate animal breeding and time to reach specific developmental stages are required as well as the cost for maintenance of animals. Despite this inconvenience and cost, primary cultured neurons have a key role in neuroscience research. Neuronal cell lines, such as PC12 and Neuro-2a cells, are also widely used to study neuronal differentiation, neurite growth, and cell toxicity as neuronal cell models *in vitro* (Calderon et al., 1999; Greene and Tischler, 1976; Klebe and Ruddle, 1969; Prasad, 1975; Tremblay et al., 2010). Neuronal cell lines express several synaptic proteins and neuronal transmitters, and under certain conditions synapse-like structures are formed (Amino et al., 2002; Jeon et al., 2010; Tremblay et al., 2010). However, as neuronal cell lines do not form functional synapses, they are not well-suited for the study of synapse formation, synaptic activity, and synaptic plasticity.

There has been great interest in recent years to develop methods for cryopreservation of primary neuronal cultures in a way that preserves the morphology and function seen in freshly prepared cultures (Das et al., 1983; Fang and Zhang, 1992; Higgins et al., 2011; Luyet and Gonzales, 1953; Negishi et al., 2002; Paynter, 2008; Robert et al., 2016). A high-quality bank of cryopreserved neurons would provide flexibility in experimental design, increase reproducibility, and facilitate collaboration among laboratories. In recent years, several companies have started to offer both cryopreserved neurons and freezing media. In addition, recent studies showed the improvement of cryopreservation methods for primary neurons by using newly developed, commercially available freezing media (Parker et al., 2018; Pischedda et al., 2018). However, the viability of cryopreserved neuronal cultures is commonly reduced compared to freshly prepared neurons and the contents of commercial freezing media are undisclosed.

Here, we report a simple method for the use of DMSO as a cryoprotectant for the long-term preservation of primary rat hippocampal and cortical neuronal cultures. This method of cryopreservation does not involve use of expensive commercial or proprietary materials. We thawed cryopreserved neurons after more than 1 year of storage in liquid nitrogen and assessed their viability, development, synaptogenesis, and stimulus-responsiveness by combing cell imaging, pharmacological treatments and biochemical signaling assays. Our DMSO-based method for cryopreservation provides neuronal cultures that are developmentally and functionally similar to freshly prepared cultures.

2. Materials and methods

2.1. Preparation of coverslip and culture dish for primary neuronal cultures

Coverslips (Marienfeld-Superior, Lauda-Königshofen, Germany) were put into a 50-ml conical tube to which 1 N HNO_3 (Sigma-Aldrich, St. Louis, MO) was added, then sonicated for 15 min in ultrasonic bath (SONOREX TK20; BANDLIN electronic GmbH & Co KG, Berlin, Germany). After 4 times washing with Milli-Q® water (Merck, Darmstadt, Germany), the coverslips were sonicated again for 15 min. After washing once again, the coverslips were autoclaved at 121 °C for 20 min. Then, the coverslips were treated with absolute ethanol, and dried in a biological safety cabinet. The coverslips were stored with a desiccant in a lightproof box. Coverslips for immunocytochemistry and plastic culture dishes for biochemistry were coated with 1 and 0.1 mg/ml poly-L-lysine (PLL; Sigma-Aldrich) in borate buffer (Thermo Fisher Scientific, Waltham, MA) at room temperature for overnight, respectively. They were washed 3 times with Milli-Q® water followed by preincubated with plating medium, comprised of Minimum Essential Medium (MEM, Thermo Fisher Scientific), 10 % FBS (Sigma-Aldrich), 0.6 % glucose (Sigma-Aldrich), and 1 mM sodium pyruvate (Thermo Fisher Scientific), and 100 U/ml Penicillin-Streptomycin (Thermo Fisher Scientific) for equilibration at 37 °C in a humidified incubator with 5 % CO_2.

2.2. Primary neuronal culture

All animal procedures were performed in accordance with the Norwegian animal care committee regulations. Hippocampal and cortical neuronal cultures were prepared according to previously described methods with several modifications (Ishizuka et al., 2014; Kaech and Banker, 2006). Time-mated pregnant Wistar rats were deeply anesthetized and euthanized by CO_2. Embryonic day 18.5 (E18.5) fetuses were removed from the uterus. Then, their brains were dissected out and placed them in a 10-cm dish containing ice-cold tissue buffer, comprised of Hank's Balanced Salt Solution (HBSS), 10 mM Hepes (Thermo Fisher Scientific), and 100 U/ml Penicillin-Streptomycin in a laminar flow hood. The meninges were removed using a dissecting microscope, then hippocampal and cortical tissues were dissected. The brains and tissues were kept in ice-cold tissue buffer throughout the procedure. For cell dissociation, the tissues were placed in a 15-ml conical centrifuge tube, and treated with 0.05 % Trypsin-EDTA solution (Thermo Fisher Scientific) containing 10 mM Hepes and 100 U/ml Penicillin-Streptomycin at 37 °C for 10 min in a water bath. Trypsin solution was gently removed using a Pasteur pipette, then tissues were incubated with 5 ml of tissue buffer at room temperature for removing trypsin from the tissues. After washing twice, the tissues were brought 2 ml of plating medium. Then, they were mechanically dissociated by repeatedly pipetting them up and down in a Pasteur pipette: Firstly, the tissues were passed through a regular sterilized Pasteur pipet for 10 times, then continued with 5–10 passes through a flame-polished Pasteur pipette. The cell suspensions were seeded at 10,000–15,000 cells/cm^2 on PLL-coated 18 mm coverslips in 12-well plates for immunocytochemistry, and at 40,000–60,000 cells/cm^2 on PLL-coated 6 cm culture dishes or 6-well culture plate for biochemistry, respectively. The cells were maintained in plating media for 2–3 h. After attachment of the cells, the media were replaced to maintenance media, comprised of Neurobasal™ Medium (Thermo Fisher Scientific), 2 % B-27™ supplement (Thermo Fisher Scientific), and 0.25 % GlutaMAX™-I (Thermo Fisher Scientific). At 4 DIV, 1 μM cytosine arabinoside (AraC, Sigma-Aldrich) was added to the cultures for inhibiting glial proliferation. All experiments were performed using cryopreserved neurons stored for 12–18 months in liquid nitrogen.

2.3. Freezing and thawing neurons

To cryopreserve hippocampal and cortical neurons, 500 μl of freezing media (80 % FBS + 20 % DMSO) was added into the 2 ml cryovials (Corning, NY). Then, 500 μl of cell suspension (in plating medium) was added to cryovials and gently mixed. The final concentration of FBS and DMSO were 45 % and 10 %, respectively because plating medium also contains 10 % FBS. The remaining 45 % volume in cryovial was derived from the cells and other components of plating media. The cryovials were placed in a cell freezing container (CoolCell™ LX cell freezing container, Corning), stored at −80 °C (−1 °C/min) for 4 h, and then transferred to the liquid phase of liquid nitrogen for long-term storage, more than 1 year. To thaw, the cryovials were rapidly thawed at 37 °C in a water bath until a small ice crystal remained, then 1 ml of warm plating medium was gently added to the cryovials. The cell suspension was gently transferred to a 50-ml conical tubes containing 8 ml of plating medium. The cell suspension was not centrifuged at any time in order to avoid cellular damage associated with centrifugation and resuspension of cell pellets. After counting the cell density, the cells were seeded on coverslips in 12-well plates and culture dishes at an appropriate density. After 2–3 h, plating media were changed to maintenance media as done for freshly prepared neurons.

2.4. Pharmacological treatment

After incubation for 21 DIV, the cells were pharmacologically treated, and then analyzed biochemically and immunocytochemically.

Table 1
Primaryantibody source and usage.

Target	Source	RRID	Dilution	Figure
Drebrin	Rb polyclonal, Bethyl (A304-517A)	AB_2620711	1:1000	2
Neurofilament H, phospho	Ms monoclonal, Covance Research Products Inc (SMI-310R-100, SMI-310)	AB_509989	1:100	2
Drebrin	Ms monoclonal, Abcam (ab12350, M2F6)	AB_299034	1:400	4, 5
MAP2	Ck polyclonal, EnCor Biotechnology (CPCA-MAP2)	AB_2138173	1:3000	5
Synapsin I	Rb polyclonal, Millipore (AB1543P)	AB_90757	1:1000 (ICC)	4
			1:2000 (WB)	6
VGLUT1	Rb polyclonal, Synaptic Systems (135 303)	AB_887875	1:500	4
PSD95	Ms monoclonal, Thermo Fisher Scientific (MA1-046, 7E3-1B8)	AB_2092361	1:100 (ICC)	4
			1:200 (WB)	6
Homer 1a	Rb polyclonal, Synaptic Systems (160 013)	AB_2619856	1:2000	6
NMDAR2A	Rb polyclonal, Thermo Fisher Scientific (OPA1-04021)	AB_2112313	1:500	6
NMDAR2B	Rb polyclonal, Abcam (ab65783)	AB_1658870	1:1000	6
GluA1	Rb monoclonal, Millipore (04-855, C3T)	AB_1977216	1:1000	6
GluA2	Ms monoclonal, Millipore (MAB397, 6C4)	AB_2113875	1:1000	6
Synaptotagmin I	Ms monoclonal, Santa Cruz Biotechnology (sc-136480, 15)	AB_10611453	1:500	6
Matrin 3	Rb polyclonal, Bethyl (A300-591A)	AB_495514	1:3000	6
Vimentin	Rb polyclonal, Synaptic Systems (172 002)	AB_887887	1:2000	6
CaMKIIβ	Ms monoclonal, Thermo Fisher Scientific (13-9800, CB-beta-1)	AB_2533045	1:500	6
mTOR	Rb polyclonal, Cell Signaling Technology (2972)	AB_330978	1:500	6
p44/42 MAPK (Erk1/2)	Rb polyclonal, Cell Signaling Technology (9102)	AB_330744	1:2000	6, 7
GAPDH	Ms monoclonal, Thermo Fisher Scientific (MA5-15738, GA1R)	AB_10977387	1:3000	6
β-actin	Ms monoclonal, Sigma-Aldrich (A1978, AC-15)	AB_476692	1:5000	6, 7
Arc	Ms monoclonal, Santa Cruz Biotechnology (sc-17839, C-7)	AB_626696	1:200	7
p44/42 MAPK (Erk1/2), phospho (Thr202/Tyr204)	Rb monoclonal, Cell Signaling Technology (4370, D13.14.4E)	AB_2315112	1:2000	7

Rb: rabbit, Ms: mouse, Ck: chicken.

For treatment with brain-derived neurotrophic factor (BDNF), freshly prepared and cryopreserved cortical neurons were seeded on 6-well plates. Cultures were treated with 50 ng/ml human BDNF (Alomone Labs, Jerusalem, Israel) for 1, 2, and 4 h followed by western blotting. For treatment with glutamate, freshly prepared and cryopreserved hippocampal neurons were seeded on PLL-coated coverslips and treated with 50 μM glutamate (Sigma-Aldrich) for 10 min followed by immunocytochemistry.

2.5. Transfection

For dendritic spine density analysis, the hippocampal neurons were transfected with pEGFP-N1 plasmid vector (Clontech, Palo Alto, CA) using Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific) at 9–10 DIV. Briefly, 400 μl/well of transfection media, Neurobasal™ Medium supplied with 0.25 % GlutaMAX™-I, were added in new 12-well plate (transfection plate), and equivalated at 37 ˚C in a humidified incubator with 5 % CO_2. Then, 0.5 μg/well of pEGFP-N1 vector and 1 μl/well of Lipofectamine 2000 Reagent were diluted in 50 μl/well of transfection media in separate tubes. After 5 min incubation at room temperature, they were mixed, and incubated for 30 min at room temperature. After the incubation, the coverslips were transferred from culture plate to transfection plate. Then, the cells in transfection plate were added with 100 μl/well of DNA-Lipofectamine 2000 reagent mixtures and incubated for 45 min at 37 ˚C in a humidified incubator with 5 % CO_2. After the incubation, the coverslips were put back to original culture plate. The neurons were maintained at 37 ˚C in a humidified incubator with 5 % CO_2.

2.6. Cell viability assay

The cell viability was measured immediately after post-thaw by the trypan blue exclusion method. After thawing the cryopreserved neurons, the cell suspension and Trypan Blue Dye 0.4 % solution (Bio-Rad Laboratories, Inc., Hercules, CA) were mixed in equal amounts. Then, the mixture was loaded onto a cell counting slide (Counting Slides, Dual Chamber for Cell Counter, (Bio-Rad Laboratories, Inc.), and cell viability was measured using a TC20™ Automated Cell Counter (Bio-Rad Laboratories, Inc.). The WST-1 (sodium 5-(2,4-disulfophenyl)-2-(4-iodophenyl)-3-(4-nitrophenyl)-2H tetrazolium inner salt) assay was developed and performed to measure the viability of cell culture (Berridge et al., 2005; Ishiyama et al., 1995, 1993). Both freshly prepared and cryopreserved hippocampal neurons were seeded in a 96-well plate at 10,000 cells/well in a final volume of 100 μl/well of plating media. After 2–3 h incubation, the media were changed to maintenance media containing 1 μM of AraC, then maintained until 1, 3, 5, 7, and 14 DIV. Cultured neurons were added with 10 μl/well of Cell Proliferation Reagent WST-1 (Sigma-Aldrich) and incubated for 1.5 h at 37 ˚C. Then, the absorbance was measured at 440 nm using a micro plate reader (SpectraMax® Plus 384, Molecular Devices, San Jose, CA). The viability of freshly prepared neurons was shown as control (100 %) at each experiment day (1, 3, 5, 7, and 14 DIV). Phase contrast imaging was performed on Nikon Eclipse TE2000-E inverted microscopy (Nikon Corporation, Tokyo, Japan). Samples were imaged using a Plan Fluor ELWD 20x/0.45 NA objective and a DS-Qi1 monochrome digital camera controlled by NIS Elements software (Nikon corporation).

2.7. Immunocytochemistry and AM1-43 staining

Cultured hippocampal neurons plated on PLL-coated coverslips were fixed with 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 min at room temperature. After permeabilization with 0.1 % Triton X-100 in PBS (pH 7.4), the neurons were incubated with 3 % BSA in PBS for 1 h at room temperature, and then incubated with primary antibodies at 4 ˚C for overnight. After washing coverslips 5 times with PBS, the cells were incubated with appropriate secondary antibodies for 1 h at room temperature. After washing coverslips 5 times with PBS, the coverslips were mounted in Prolong Diamond Antifade mounting media (Thermo Fisher Scientific). The primary antibodies used in this study are described in Table 1. The secondary antibodies are as follows: Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (Thermo Fisher Scientific Cat# A-11008, RRID:AB_143165, 1:500), Goat anti-Rabbit IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (Thermo Fisher Scientific Cat# A-21245, RRID:AB_2535813, 1:500), Goat anti-Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 (Thermo Fisher Scientific Cat# A-11031, RRID:AB_144696, 1:500), Donkey Anti-Chicken IgY

(IgG) (H + L) Antibody, CF488A Conjugated (Biotium Cat# 20166, RRID:AB_10854387, 1:500). Fluorescently-conjugated phalloidin (Alexa Fluor® 647 phalloidin, Thermo Fisher Scientific Cat# A22287, RRID:AB_2620155, 1:100) was used to label filamentous actin (F-actin). For staining recycled synaptic vesicles, hippocampal neurons were stimulated with depolarizing K^+ buffer containing 77.5 mM KCl, 47.5 mM NaCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 20 mM Hepes, 15 mM glucose, and 0.01 mM glycine (pH 7.4) (Taylor and Hewett, 2002) in the presence of 4 µM AM1-43 (Biotium, Inc. Fremont, CA) for 1 min followed by PFA fixation and F-actin staining. Fluorescence imaging was performed on AxioImager Z1 microscopy (Carl Zeiss AG, Oberkochen, Germany). Samples were imaged using Plan-ApoChromat 20x/0.75 NA (Carl Zeiss AG), EC plan-Neofluar 40x/1.30 Oil M27 (Carl Zeiss AG), and Plan-ApoChromat 63x/0.140 Na Oil DIC M27 (Carl Zeiss AG) objectives, and Axiocam 503 mono digital camera controlled by ZEN pro software (Carl Zeiss AG). The captured fluorescence images were analyzed by using MetaMorph® software (Molecular Devices).

2.8. Staging of early-developmental neurons

Both freshly prepared and cryopreserved hippocampal neurons were classified into 3 neuronal developmental stages: stage 1, stage 2, and stage 3 according to previously described method (Ohara et al., 2015). Cultured neurons at 1 DIV were immunostained with anti-drebrin antibody (Bethyl Laboratories, Inc. Montgomery, TX), anti-phosphorylated-neurofilament H & M (p-NF) antibody (BioLegend, San Diego, CA), and Alexa Fluor® 647 Phalloidin (Thermo Fisher Scientific). Neurites were defined as a process extending more than 10 µm from the soma. An axon was defined as a neurite labeled by anti-p-NF antibody. We defined early-developmental stages as stage 1, a neuron with lamellipodia without neurites; stage 2, a neuron with some neurites but no axon; stage 3, a cell with an axon.

2.9. Assessment of synapse formation

Hippocampal neurons transfected with EGFP at 21 DIV were subjected to dendritic spine and protein cluster density analyses. Dendritic protrusions > 0.5 and < 8 µm length were analyzed as dendritic spine according to previous study (Ishizuka et al., 2014). One to three dendrites from 1 neuron were randomly selected and the density was measured. Synapsin I, drebrin, vesicular glutamate transporter 1 (VGLUT1), postsynaptic density protein 95 (PSD95), and AM1-43 clusters were defined as a round immunofluorescent region with a peak fluorescence intensity two-fold greater than the average fluorescence intensity of dendrites, and were counted along the dendrite according to previous study (Ishizuka et al., 2014). Both dendritic spine density and protein cluster analyses were performed by using MetaMorph® software.

2.10. Electrophoresis and western blotting

SDS-PAGE and western blotting were performed as described previously (Ishizuka et al., 2014). Cells were lysed in lysis buffer, containing 10 mM Tri − HCl (pH 7.4), 150 mM NaCl, 2 % SDS, 20 mM NaF, 1 mM Na_3VO_4, and cOmplete™, EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich). After centrifugation, supernatants were collected, and protein concentration were determined using Micro BCA Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of protein were subjected to SDS-PAGE and transferred to PVDF membrane (Thermo Fisher Scientific). The membranes were incubated with the appropriate primary and secondary antibodies after incubation with 10 % BSA in TBS-T at room temperature for 1 h. The primary antibodies are given in Table 1. The secondary antibodies were Goat Anti-Mouse IgG, H & L Chain Antibody, Peroxidase Conjugated (Merck, Cat# 401253, RRID:AB_437779) and Goat Anti-Rabbit IgG, H & L Chain Specific Peroxidase Conjugate antibody (Merck Cat# 401315, RRID:AB_2617117). Peroxidase activity was detected using chemiluminescence regent (Clarity Western ECL substrate, Bio-Rad Laboratories, Inc.) and visualized by an image analyzer using Image Lab™ Software (Gel Doc™ XR + , Bio-Rad Laboratories, Inc.). Quantification of western blotting and figure presentation were performed using ImageJ/FIJI (RRID: RRID:SCR_002285)

2.11. Preparation of synaptoneurosomes

The synaptoneurosome fraction was isolated as previously described (Villasana et al., 2006) with minor modifications for cultured neurons. Briefly, both freshly prepared and cryopreserved cortical neurons seeded on 6-cm dishes were harvested in synaptoneurosome buffer (pH 7.0), containing 10 mM Hepes, 1 mM EDTA, 2 mM EGTA, 0.5 mM DTT, and cOmplete™ at 4 °C using cell scrappers. Cells were homogenized by using 1 ml disposable syringe (Omnifix® -F, B. Braun Medical AS, Norway) with 25 G needle (Becton Dickinson, Franklin Lakes, NJ) for 3 times. The cell homogenate sample was set aside for western blotting as total homogenate. From this step forward the homogenate was always kept ice-cold to minimize proteolysis throughout the isolation procedure. The cell lysates were loaded into 1 ml disposable syringes and filtered twice through three layers of a prewetted 100 µm pore nylon net filter (Merck) held in 13 mm diameter filter holders (Swinnex Filter Holder, Merck). Then, the filtrated samples were loaded into 1 ml disposable syringes again and filtered through a pre-wetted 5 µm pore hydrophilic filter (Durapore® Membrane Filter, Merck) held in 13 mm diameter filter holders. The filtered samples were centrifuged at 1,000 × g for 20 min. The supernatant was used for cytosolic fraction. The pellet obtained corresponded to the synaptoneurosomal compartment. The isolated synaptoneurosomes were resuspended in lysis buffer.

2.12. Statistics

Statistical analyses were performed using GraphPad Prism ver. 7.05 for Windows (GraphPad Software, San Diego, CA) and EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria, version 3.5-2). More precisely, it is a modified version of R commander (version 2.5-1) designed to add statistical functions frequently used in biostatistics (Kanda, 2013). For continuous variables, normality tests were performed to determine data distribution before choosing parametric or nonparametric analyses. Box-and-whisker plots were used for showing all individual data points. The data shown in box-and whisker plot were subjected to median comparison test. Bar graphs were used for showing the mean value. For ordinal variables, chi-square test was used to determine relationship between cell type (fresh preparation or cryopreservation) and cell development. Aligned rank transform (ART) was performed in order to apply non-Gaussian distribution data to parametric two-factor factorial analysis of variance (ANOVA) (Wobbrock et al., 2019). Statistical significance was set at $p < 0.05$. The detailed information of statistical analysis is described in Table 2.

3. Results

3.1. Neuronal morphology and viability of cryopreserved hippocampal neurons are equivalent to freshly prepared neurons

To evaluate the viability of cryopreserved primary neurons, we dissociated hippocampi from E18.5 rats and divided into two groups: freshly prepared and cryopreserved neurons. The cell suspension for cryopreservation was mixed with freezing medium containing 80 % FBS and 20 % DMSO at a 1:1 ratio in a cryovial. The vials were stored at –80 °C in controlled condition (–1 °C/min) for 4 h followed by long-term storage in liquid nitrogen. After thawing, the cells were seeded on 96-well plates at 10,000 cells/well same as freshly prepared neurons.

Table 2
Statistics table.

	Figure	Data structure	Type of test	Comparison group	*p* value
a	1B	Non-Gaussian distribution	Kolmogorov-Smirnov test	Fresh vs. Cryo. (post-thaw)	0.4740
b	1C	Gaussian distribution	Unpaired parametric Welch's *t* test	Fresh vs. Cryo. (1 DIV)	0.6955
c	1C	Gaussian distribution	Unpaired parametric Welch's *t* test	Fresh vs. Cryo. (3 DIV)	0.3087
d	1C	Gaussian distribution	Unpaired parametric Welch's *t* test	Fresh vs. Cryo. (5 DIV)	0.3965
e	1C	Gaussian distribution	Unpaired parametric Welch's *t* test	Fresh vs. Cryo. (7 DIV)	0.1244
f	1C	Gaussian distribution	Unpaired parametric Welch's *t* test	Fresh vs. Cryo. (14 DIV)	0.2504
g	2C	N/A	Chi-square test	2 × 3 table	0.1376
h	3B	Gaussian distribution	Mann-Whitney *U* test	Fresh vs. Cryo.	0.3279
i	4B	Gaussian distribution	Mann-Whitney *U* test	Fresh vs. Cryo. (Synapsin I)	0.2882
j	4B	Gaussian distribution	Mann-Whitney *U* test	Fresh vs. Cryo. (Drebrin)	0.4397
k	4D	Gaussian distribution	Mann-Whitney *U* test	Fresh vs. Cryo. (VGLUT)	0.0666
l	4D	Gaussian distribution	Mann-Whitney *U* test	Fresh vs. Cryo. (PSD95)	0.7025
m	4F	Gaussian distribution	Mann-Whitney *U* test	Fresh vs. Cryo. (AM1-43)	0.7450
n	5B	Non-Gaussian distribution	Two-factor factorial ANOVA after ART*	ANOVA (interaction)	0.7516
o	5B	Non-Gaussian distribution	Brunner-Munzel test	Fresh: Ctrl vs. Glu stim.	< 0.0001
p	5B	Non-Gaussian distribution	Brunner-Munzel test	Cryo.: Ctrl vs. Glu stim.	< 0.0001
q	6C	Non-Gaussian distribution	Permuted Brunner-Munzel test	Fresh vs. Cryo. (PSD95)	0.8
r	6C	Non-Gaussian distribution	Permuted Brunner-Munzel test	Fresh vs. Cryo. (Homer1a)	0.2
s	6C	Non-Gaussian distribution	Permuted Brunner-Munzel test	Fresh vs. Cryo. (NMDAR2A)	0.4857
t	6C	Non-Gaussian distribution	Permuted Brunner-Munzel test	Fresh vs. Cryo. (NMDAR2B)	1
u	6C	Non-Gaussian distribution	Permuted Brunner-Munzel test	Fresh vs. Cryo. (GluA1)	0.4286
v	6C	Non-Gaussian distribution	Permuted Brunner-Munzel test	Fresh vs. Cryo. (GluA2)	1
w	6C	Non-Gaussian distribution	Permuted Brunner-Munzel test	Fresh vs. Cryo. (Syt I)	0.2
x	6C	Non-Gaussian distribution	Permuted Brunner-Munzel test	Fresh vs. Cryo. (Syn I)	0.8
y	6C	Non-Gaussian distribution	Permuted Brunner-Munzel test	Fresh vs. Cryo. (Matrin 3)	0.2
z	6C	Non-Gaussian distribution	Permuted Brunner-Munzel test	Fresh vs. Cryo. (Vimentin)	0.8
aa	6C	Non-Gaussian distribution	Permuted Brunner-Munzel test	Fresh vs. Cryo. (CaMKIIβ)	0.4857
ab	6C	Non-Gaussian distribution	Permuted Brunner-Munzel test	Fresh vs. Cryo. (mTOR)	1
ac	6C	Non-Gaussian distribution	Permuted Brunner-Munzel test	Fresh vs. Cryo. (Erk1/2)	0.8
ad	6C	Non-Gaussian distribution	Permuted Brunner-Munzel test	Fresh vs. Cryo. (GAPDH)	0.5143
ae	6C	Non-Gaussian distribution	Permuted Brunner-Munzel test	Fresh vs. Cryo. (β-actin)	0.3429
af	7B	Gaussian distribution	Two-factor factorial ANOVA	ANOVA (interaction)	0.4977
ag	7B	Gaussian distribution	Dunnet's test	Fresh: Ctrl vs. BDNF 1h	0.7787
ah	7B	Gaussian distribution	Dunnet's test	Cryo.: Ctrl vs. BDNF 1h	0.4506
ai	7B	Gaussian distribution	Dunnet's test	Fresh: Ctrl vs. BDNF 2h	0.0024
aj	7B	Gaussian distribution	Dunnet's test	Cryo.: Ctrl vs. BDNF 2h	< 0.0001
ak	7B	Gaussian distribution	Dunnet's test	Fresh: Ctrl vs. BDNF 4h	0.4525
al	7B	Gaussian distribution	Dunnet's test	Cryo.: Ctrl vs. BDNF 4h	0.1426
am	7C	Gaussian distribution	Two-factor factorial ANOVA	ANOVA (interaction)	0.9639
an	7C	Gaussian distribution	Dunnet's test	Fresh: Ctrl vs. BDNF 1h	0.0004
ao	7C	Gaussian distribution	Dunnet's test	Cryo.: Ctrl vs. BDNF 1h	0.0007
ap	7C	Gaussian distribution	Dunnet's test	Fresh: Ctrl vs. BDNF 2h	0.0033
aq	7C	Gaussian distribution	Dunnet's test	Cryo.: Ctrl vs. BDNF 2h	0.0013
ar	7C	Gaussian distribution	Dunnet's test	Fresh: Ctrl vs. BDNF 4h	0.1179
as	7C	Gaussian distribution	Dunnet's test	Cryo.: Ctrl vs. BDNF 4h	0.0446

* ART: Aligned Rank Transform.

Bright-field images of freshly prepared neurons and cryopreserved neurons were acquired at 7 DIV. The cryopreserved hippocampal neurons formed a complex network of neurites same as freshly prepared hippocampal neurons (Fig. 1A). We also evaluated the cell viability of cryopreserved neurons by trypan blue exclusion method and WST-1 assay. The viability of cryopreserved cultures was comparable to freshly prepared culture during development (1–14 DIV) (Fig. 1B and C). These results suggest that cell viability and neurite outgrowth complexity is similar between DMSO-cryopreserved and freshly prepared neuronal cultures.

3.2. Initial maturation of cryopreserved hippocampal neurons is equivalent to freshly prepared neurons

Next, we evaluated the maturation of hippocampal neurons according to three defined developmental stages (See Materials and methods: **2.8. Staging of early-developmental neurons**). Cultured neurons show unique structures, such as lamellipodia, several minor processes, and putative axon, during their early development. Cells at 1 DIV were imaged for the analysis of initial morphological maturation. We used immunocytochemical staining and examined subcellular localization of drebrin, F-actin and p-NF (Fig. 2A). Drebrin, **d**evelopmentally **re**gulated **br**ain prote**in**, is an actin-binding protein, and shows a characteristic distribution pattern during early neuronal development. In stage 1 neurons, drebrin and F-actin were distributed to lamellipodia surrounding cell body both in freshly prepared and cryopreserved neurons. In stage 2 neurons with minor processes, drebrin and F-actin localized to the tips of short minor processes. In stage 3 neurons, p-NF immunoreactivity was detected only in the longest process; this process is destined to become the axon in future (gray scale images in Fig. 2A) (Dotti et al., 1988). In addition, we compared the subcellular localization of drebrin, F-actin, and p-NF in axonal growth cones. As shown in Fig. 2B, in line with previous studies (Mizui et al., 2009; Ohara et al., 2015), F-actin was distributed in the tips of growth cones; drebrin is localized in the middle of growth cones. On the other hand, the expression of p-NF was mainly detected in axonal process. Cryopreserved hippocampal neurons apparently show normal initial maturation same as freshly prepared neurons. For quantitative analysis, we totally classified 1,755 of freshly prepared cells and 1,539 of cryopreserved cells from 3 independent preparations into stage 1–3 according to previous study (Ohara et al., 2015). The percentages of stage 1–3 cells in freshly prepared neurons were 43.43 % (stage 1), 52.53 % (stage 2), and 4.04 % (stage 3), respectively. The percentage of stage 1–3 cells in cryopreserved cultures were 46.47 %

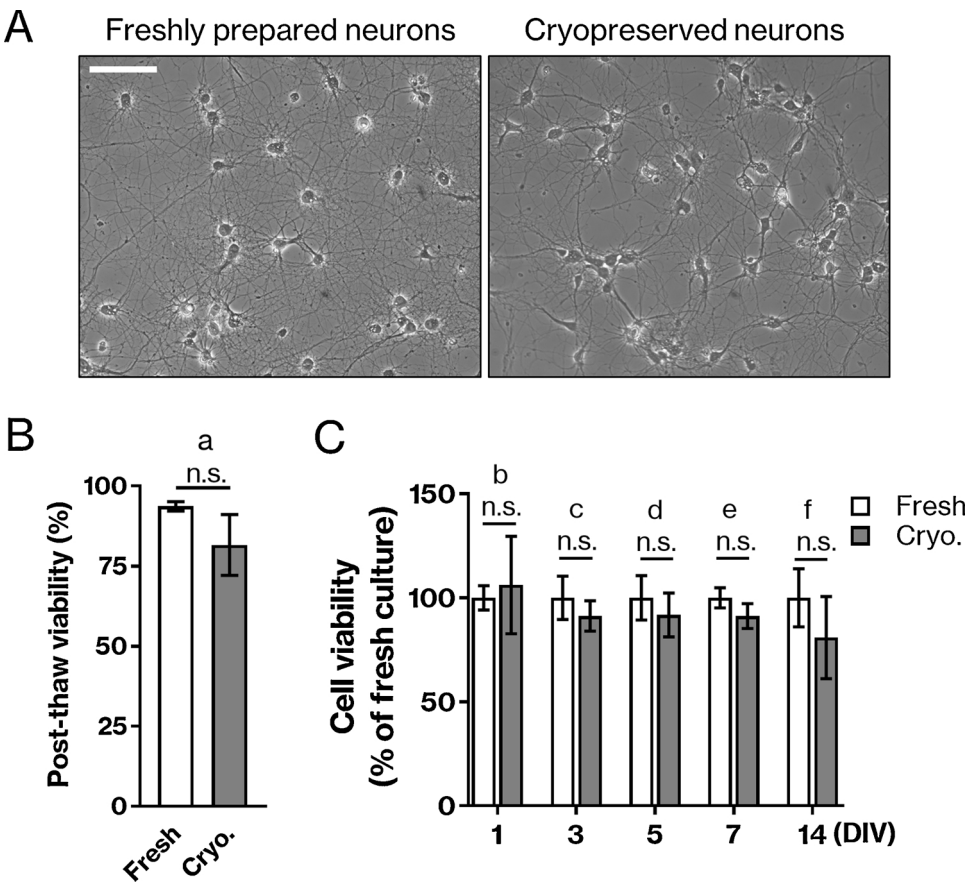


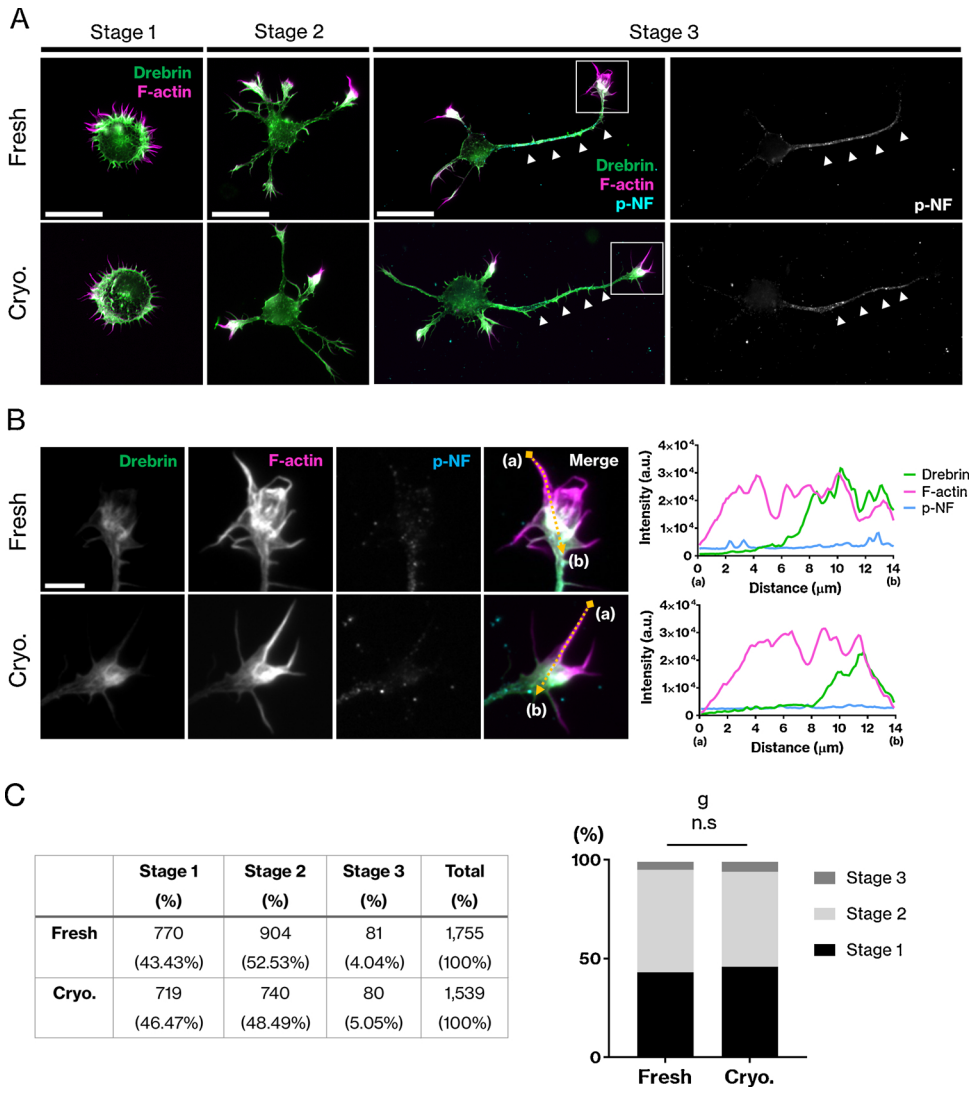
Fig. 1. There is no difference in cell viability between freshly prepared and cryopreserved neurons. ***A,*** Both freshly prepared and cryopreserved hippocampal neurons were concurrently plated and then imaged by Nikon Eclipse TE2000-E inverted microscopy at 7 DIV. Scale bar = 50 μm. ***B,*** The viability of cells immediately post-thaw was measured using the trypan blue exclusion method and automated cell counter. Data are expressed as mean ± standard error of the mean (SEM). $N = 6$ different preparations. $p = 0.4740$[a], n.s. = non-significant. ***C,*** The viability of both freshly prepared and cryopreserved neurons was measured by WST-1 assay on indicated DIV. Date are expressed as mean ± standard error of the mean (SEM). $N = 3$, $p = 0.6955$[b]; $p = 0.3087$[c]; $p = 0.3965$[d]: $p = 0.1244$[e]; $p = 0.2504$[f], n.s. = non-significant.

(stage 1), 48.49 % (stage 2), and 5.05 % (stage 3), respectively (Fig. 2C). Statistical analysis was performed for clarifying the relationship between the effect of cryopreservation and early development of the cells. There is no significant effect of cryopreservation on cell development ($p = 0.1376$[g], Fig. 2C). These results suggest that development of DMSO-cryopreserved neurons is similar to that of freshly prepared neurons.

3.3. Synapse formation and glutamate-induced drebrin translocation in cryopreserved hippocampal neurons are equivalent to freshly prepared neurons

Next, to test the effect of cryopreservation on dendritic spine formation, we measured the density of dendritic spines in both freshly prepared and cryopreserved hippocampal neurons at 21 DIV (Fig. 3A). The density of dendritic spines was comparable between fresh and cryopreserved neurons ($p = 0.3279$[h], Fig. 3B). Mature synapses have both pre- and postsynaptic proteins. Thus, we immunostained both freshly prepared and cryopreserved hippocampal cultures with several well-established pre- and postsynaptic markers: synapsin I; drebrin; VGLUT1; and PSD95 (Fig. 4). Immunocytochemical analyses showed that neither synapsin I nor drebrin cluster density along dendrites were significantly different between freshly prepared and cryopreserved hippocampal neurons (Synapsin I, $p = 0.2882$[i]; Drebrin, $p = 0.4397$[j], Fig. 4A and B). Similarly, neither VGLUT1 nor PSD95 cluster density along dendrites were significantly different between freshly prepared and cryopreserved hippocampal neurons (VGLUT1, $p = 0.0666$[k]; PSD95, $p = 0.7025$[l], Fig. 4C and D). For the assessment of synaptic activity, functional presynaptic boutons were visualized by uptake of styryl dye AM1-43. AM1-43 dye is a fixable derivative of FM dyes which are well-known fluorescent probes for staining recycled vesicles (Furuyashiki et al., 2002; Li et al., 2004). Both freshly prepared and cryopreserved hippocampal neurons were stimulated with high potassium for 1 min (See Materials and methods: **2.7. Immunocytochemistry and AM1-43 staining**). The density of AM dye-labeled puncta was measured by cluster analysis same as synaptic proteins. As shown in Fig. 4E, AM1-43 puncta were distributed along dendrites shown by F-actin staining. There was no statistical difference between freshly prepared and cryopreserved hippocampal neurons ($p = 0.7450$[m], Fig. 4F). Therefore, cryopreserved neurons have functional presynapses similar to freshly prepared neurons. Taken together, these results suggest that cryopreserved neurons form normally excitatory synapse and act as glutamatergic excitatory neurons because drebrin accumulates at postsynapse of glutamatergic neurons.

In addition to structural analyses, we examined whether the cryopreserved hippocampal neurons are functionally equivalent to freshly prepared neurons. As mentioned above, drebrin is an actin-binding protein and forms a unique stable actin structure in mature dendritic spines (Shirao et al., 2017). It is also known that the localization of drebrin is temporally changed from dendritic spines to dendritic shafts in respond to NMDA receptor (NMDAR) stimulation. (Mizui et al., 2014; Sekino et al., 2006). Drebrin is therefore recognized as a marker of a mature postsynaptic compartment (Ishizuka et al., 2014; Mitsuoka et al., 2019; Puspitasari et al., 2016). To test NMDAR-dependent drebrin translocation, both freshly prepared and cryopreserved hippocampal neuros were stimulated with 50 μM glutamate for 10 min at 21 DIV. After fixation, the cells were double-immunostained with anti-MAP2 and anti-drebrin antibodies (Fig. 5A). Importantly, statistical analysis using two-factor factorial ANOVA showed that there was no interaction effect between cell type (freshly prepared neurons or cryopreserved neurons) and glutamate stimulation ($p = 0.7516$[n], Fig. 5B); however glutamate stimulation induced drebrin translocation from dendritic spines to dendritic shaft in both freshly prepared and cryopreserved hippocampal neurons consistent with previous studies (fresh,



	Stage 1 (%)	Stage 2 (%)	Stage 3 (%)	Total (%)
Fresh	770 (43.43%)	904 (52.53%)	81 (4.04%)	1,755 (100%)
Cryo.	719 (46.47%)	740 (48.49%)	80 (5.05%)	1,539 (100%)

Fig. 2. Developmental classification of neurons. ***A,*** Freshly prepared neurons and cryopreserved neurons were triple labeled with anti-drebrin antiboty (green), Alexa 647-conjugated phalloidin (magenta), and anti-p-NF antibody (turquoise, an axonal marker) at 1 DIV. Stage 1–3 neurons were observed in both freshly prepared neurons and cryopreserved neurons. Axons are indicated with arrowheads. Scale bar = 20 μm. ***B,*** Subcellular localization of drebrin, F-actin, and p-NF in axonal growth cones. Fluorescence images showing the distribution of drebrin (green), F-actin (magenta), and p-NF (turquoise). Quantitative profiles of the signal intensities of drebrin (green line), F-actin (magenta line), and p-NF (turquoise line). The signal intensity of each protein in the axonal growth cone was obtained by measuring the intensity along the dotted arrow that extends from the distal edge of the axonal growth cone. Scale bar = 10 μm. ***C,*** Percentages of cells in each stage at 1 DIV. We classified both freshly prepared neurons (N = 1,755 cells) and cryopreserved neurons (N = 1,539) from 3 independent cultures into stage 1–3. Percentage of each stage in fresh cultures were as follows: stage 1, 43.43 %; stage 2, 52.53 %; stage 3: 4.04 %. Percentage of each stage in cryopreserved cultures were as follows: stage 1, 46.47 %; stage 2, 48.49 %; stage 3, 5.05 %. Chi-square test was performed: p = 0.1376[g]; n.s. = non-significant.

***p < 0.0001[c]; cryo., ***p < 0.0001[p]). The analysis of glutamate-induced drebrin translocation did not show any differences between freshly prepared and cryopreserved neurons. Therefore, this result suggests that cryopreserved neurons are comparable to freshly prepared neurons functionally.

3.4. The expression and localization of synaptic proteins in cryopreserved cortical neurons are comparable to freshly prepared neurons

Next, we investigated the expression of major pre- and postsynaptic proteins in synaptoneurosomes by western blotting in freshly prepared and cryopreserved cortical neurons at 21 DIV. Flow charts depicting

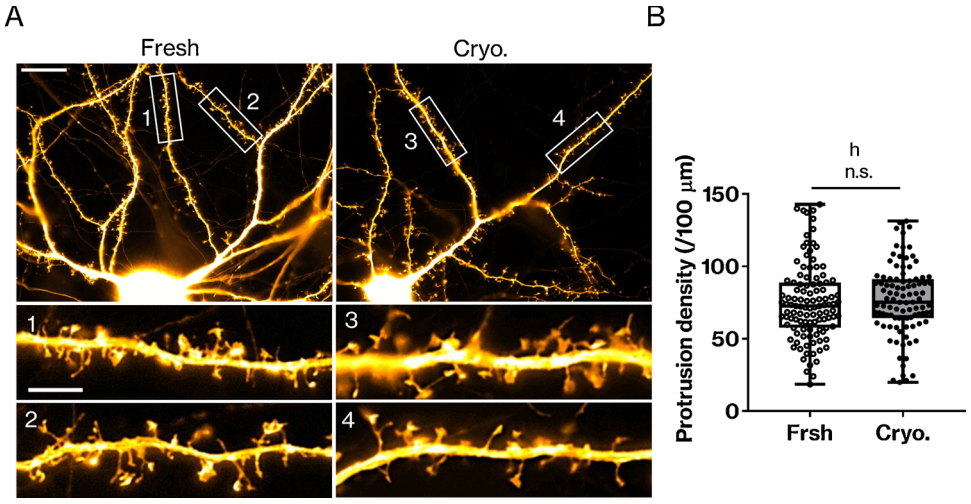


Fig. 3. Cryopreservation does not affect the formation of dendritic spines. ***A,*** Representative GFP fluorescence images of freshly prepared and cryopreserved hippocampal neurons. GFP-transfected neurons were fixed at 21 DIV. Lower images are high magnification images of the area in the rectangle in the upper images. ***B,*** Quantification of the density of dendritic spines. Box-and whisker plots show all data points from 3 independent experiments. Scale bar in low magnification images = 20 μm, Scale bar in high magnification images = 5 μm. N = 111 dendrites from 59 of freshly prepared hippocampal neurons, N = 98 dendrites from 51 of cryopreserved hippocampal neurons. Mann-Whitney U test was used for comparison between freshly prepared and cryopreserved neurons (p = 0.3279[h]; n.s. = non-significant).

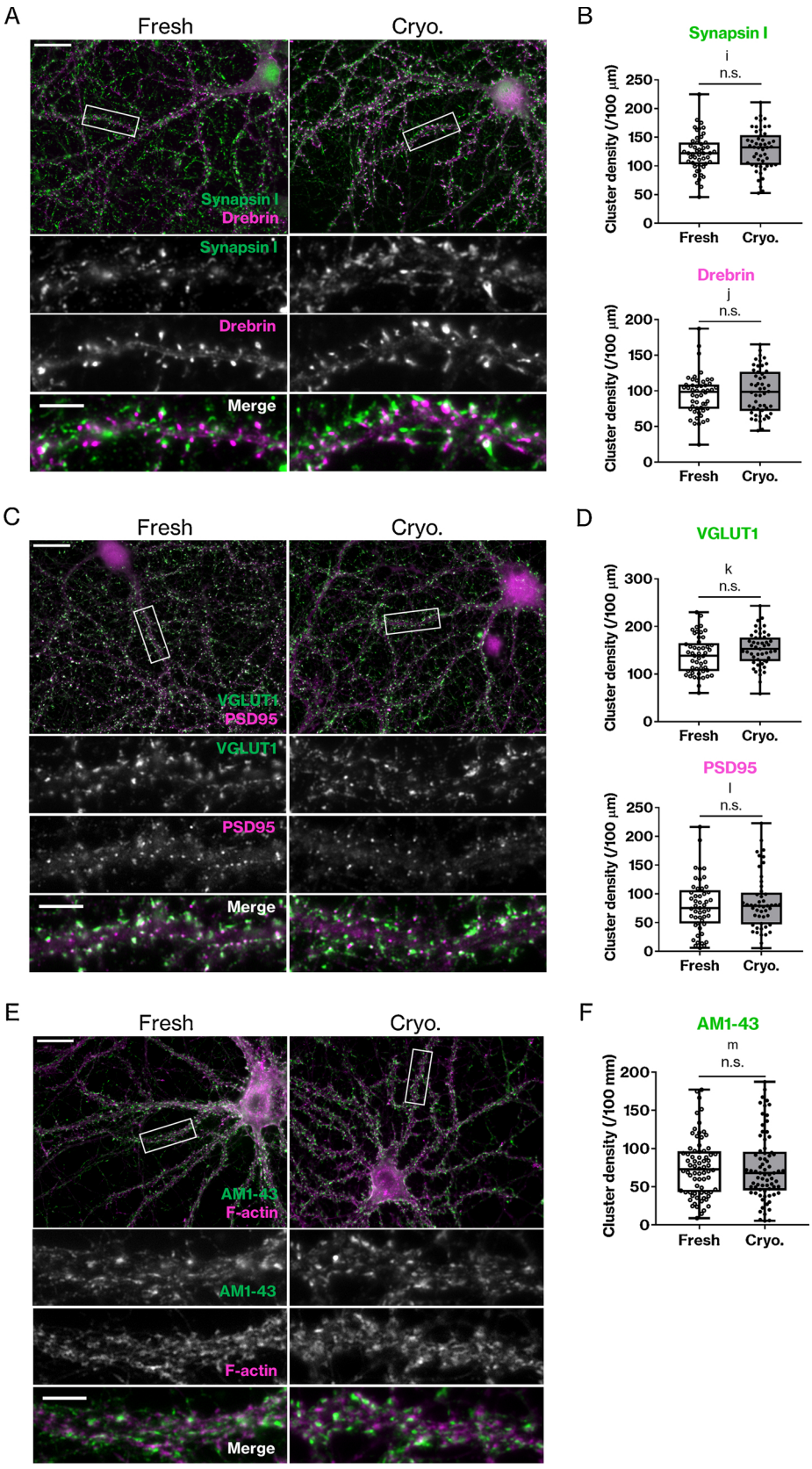


Fig. 4. Expression of pre- and postsynaptic proteins is similar in freshly prepared and cryopreserved neurons. ***A,*** Immunofluorescence images of synapsin I (presynaptic protein, green) and drebrin (postsynaptic protein, magenta). Neurons were fixed at 21 DIV and then immunostained with anti-synapsin I and anti-drebrin antibodies. Lower images are high magnification images of the area in rectangle in the upper images. ***B,*** Quantification of the densities of synapsin I and drebrin clusters. $N = 50$ cells from freshly prepared hippocampal cultures, $N = 50$ cells from cryopreserved hippocampal cultures (synapsitn I, $p = 0.2882^{i}$; drebrin, $p = 0.4397^{j}$). ***C,*** Immunofluorescence images of VGLUT1 (presynaptic protein, green) and PSD95 (postsynaptic protein, magenta). Neurons were fixed at 21 DIV and then immunostained with anti-VGLUT1 and anti- PSD95 antibodies. Lower images are high magnification images of the area in rectangle in the upper images. ***D,*** Quantification of the densities of VGLUT1 and PSD95 cluster. $N = 49$ cells from freshly prepared hippocampal cultures, $N = 49$ cells from cryopreserved hippocampal cultures (VGLUT1, $p = 0.0666^{k}$; PSD95, $p = 0.7025^{l}$). ***E,*** Fluorescence images of AM1-43 (presynaptic marker, green) and F-actin (magenta). Lower panel shows high-magnification images of the area in rectangle. ***F,*** Quantification of the density of AM1-43 cluster. $N = 72$ cells from freshly prepared hippocampal cultures, $N = 73$ cells from cryopreserved hippocampal cultures. ($p = 0.7450^{m}$). All Box-and whisker plots show all data points from 3 independent experiments. Scale bar in low magnification images = 20 µm, Scale bar in high magnification images = 5 µm. Mann-Whitney *U* test was used for comparison between freshly prepared neurons and cryopreserved neurons (n.s. = non-significant).

each procedure are shown in Fig. 6A. The cell lysates were divided into 3 fractions: total homogenate fraction (T), cytosol fraction (C), and synaptoneurosome fraction (SN). We investigated the expression of the presynaptic proteins, synaptotagmin I (Syt I) and synapsin I (Syn I), and postsynaptic proteins including NMDAR subunits (NMDAR2A, NMDAR2B), AMPA receptor subunits (GluA1, GluA2), as well as the scaffolding proteins, PSD95 and Homer1a (Fig. 6B). Quantification of immunoblots showed no significant difference in the expression of these

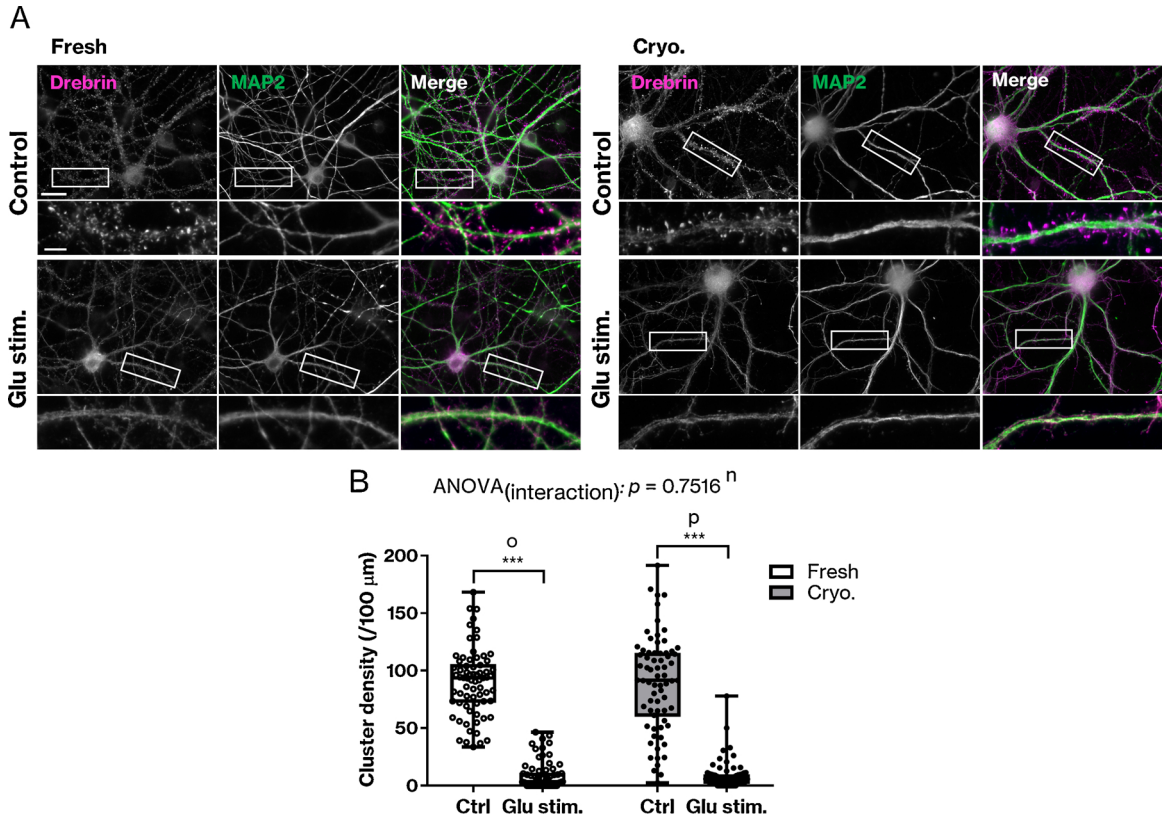


Fig. 5. Cryopreserved hippocampal have normal excitability to glutamate stimulation. ***A,*** Immunofluorescence images of MAP2 (dendrite marker, green) and drebrin (magenta). Neurons were treated with vehicle or 50 µM glutamate for 10 min at 21 DIV and then immunolabeled with anti-MAP2 and anti-drebrin antibodies. Lower images are high magnification images of the area in rectangle in the upper images. ***B,*** Quantification of the density of drebrin cluster. Box-and whisker plots show all data points from 3 independent experiments. Scale bar in low magnification images = 20 µm, Scale bar in high magnification images = 5 µm. $N = 70$ cells from freshly prepared hippocampal cultures, $N = 70$ cells from cryopreserved hippocampal cultures. Statistical comparison made by two-factor factorial ANOVA after ART for analyzing interaction effect ($p = 0.7516^{n}$) and Brunner-Munzel test for comparison between control and glutamate stimulation group in each cell type (Fresh, $***p < 0.0001^{\circ}$; Cryo., $***p < 0.0001^{p}$).

synaptic proteins in synaptoneurosomes between freshly prepared and cryopreserved cortical neurons (Fig. 6C). In addition to the synaptic proteins, we also analyzed the expression of several nuclear and cytosolic proteins in order to validate the quality of the synaptoneurosome isolation. Matrin 3, which is a nuclear matrix protein (Belgrader et al., 1991), and vimentin, which is a type III intermediate filament protein mainly expressed in astrocytes in CNS (Schnitzer et al., 1981), were hardly detected in synaptoneurosome fraction. CaMKIIβ is a cytosolic protein, but it is known that CaMKIIβ is abundantly expressed in the brain, especially in the postsynaptic density and is essential for functional and structural synaptic plasticity (Okamoto et al., 2009; Okuno et al., 2012; Peng et al., 2004). Similarly, mammalian/mechanistic target of rapamycin (mTOR) is also known to localize at synaptoneurosome, and control the translation of many hundreds of dendritic transcripts (Liao et al., 2007; Schratt et al., 2004; Takei et al., 2004). Consistent with previous studies, both CaMKIIβ and mTOR were observed in synaptoneurosome (Fig. 6B and C) In summary, similar to freshly prepared cultures, cryopreserved cultures showed equivalent enrichment of synaptic markers and depletion of a nuclear and glial marker in synaptoneurosomes, and synaptic localization of CaMKIIβ and mTOR.

3.5. BDNF-induced Erk activation and Arc upregulation are similar in cryopreserved and freshly prepared cortical neurons

Cryopreserved neurons are expected to be valuable for drug screening and analysis of signal transduction and gene expression. We assessed responsivity to BDNF. BNDF treatment is known to activate Erk1/2, and stimulate the expression of the immediate early gene product, Arc, in freshly prepared neuronal cultures (Myrum et al., 2017). Erk1/2, also known as p44/42 MAPK, are serine/threonine protein kinase that are widely expressed, and involved in many cellular functions, such as cell proliferation, differentiation, motility, and death. Many different stimuli, including BDNF, activate the Erk signaling pathway (Roskoski, 2012). Erk1/2 activation is necessary for BDNF-induced dendritic spine formation and LTP (Alonso et al., 2004; Ying et al., 2002). BDNF signaling enhances the expression of Arc in the synaptic compartment in an Erk-dependent manner (Panja et al., 2014; Yin et al., 2002). Arc synthesis is necessary for multiple forms of synaptic plasticity (Nikolaienko et al., 2018) including BDNF-induced LTP (Messaoudi et al., 2007; Ying et al., 2002). Here, both freshly prepared and cryopreserved cortical neurons were stimulated with 50 ng/ml BDNF for 1, 2, and 4 h at 21 DIV, and examined for changes in Erk1/2 phosphorylation and Arc expression. BDNF treatment resulted in upregulation of phospho-Erk1/2 and enhanced Arc expression in both cryopreserved cortical neurons and freshly prepared neurons (Fig. 7A). Importantly, no statistically significant interaction effect between cell type and BDNF stimulation was detected (Fig. 7B, Arc expression, $p = 0.4977^{af}$; Fig. 7C, Erk1/2 phosphorylation, $p = 0.9639^{am}$).

4. Discussion

4.1. Simplified DMSO-containing freezing media is suitable for long-term preservation of primary neuronal cultures

In this study, we described a simple cryopreservation protocol for

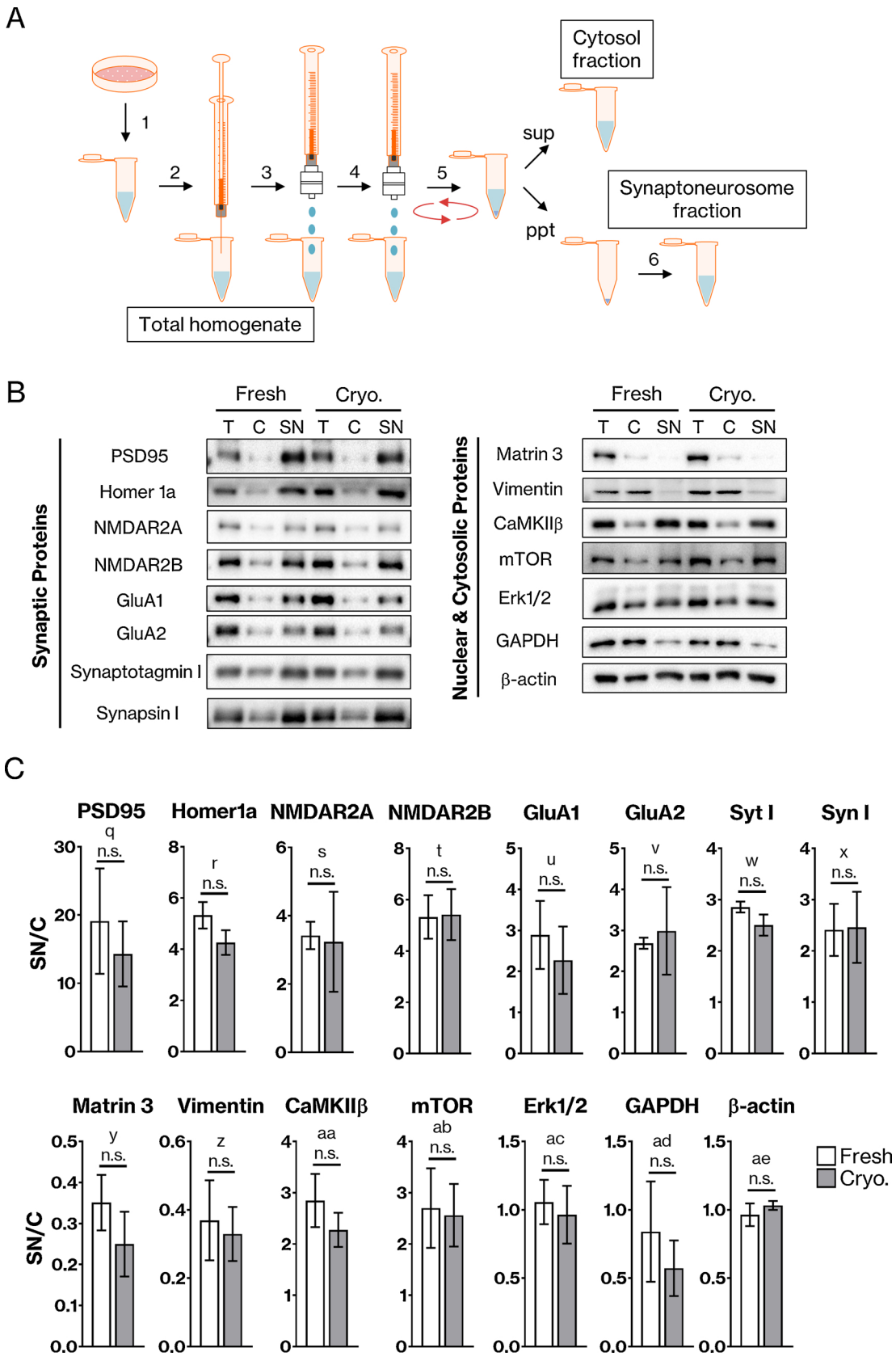


Fig. 6. Cryopreserved neurons normally express synaptic protein same as freshly prepared neurons. ***A,*** Schema of synaptoneurosome preparation. **1.** Harvest the cells by synaptoneurosome buffer; **2.** Homogenize the cells using disposable 1 ml syringe with 25 G needle for 3 times; **3.** Filtrate the cell lysate with 3 layers of prewetted 100 μm pore nylon filter twice; **4.** Filtrate the lysate through a pre-wetted 5 μm pore hydrophilic filter; **5.** Centrifuge at 1,000 × g for 20 min; **6.** lyse the pellet with cell lysis buffer. ***B,*** Typical immunoblots are shown. Left and right images are shown as major synaptic proteins and nuclear & cytosolic proteins, respectively. T: total homogenate, C: cytosol fraction, SN: synaptoneurosome fraction. ***C,*** Quantification of immunoblots. Immunoreactivity of each protein in synaptoneursomal fraction was normalized to their immunoreactivity in cytosolic fraction. Data are presented as means ± SEM, $N = 4$. Permuted Brunner-Munzel test was used for comparison between freshly prepared neurons and cryopreserved neurons (n.s. = non-significant).

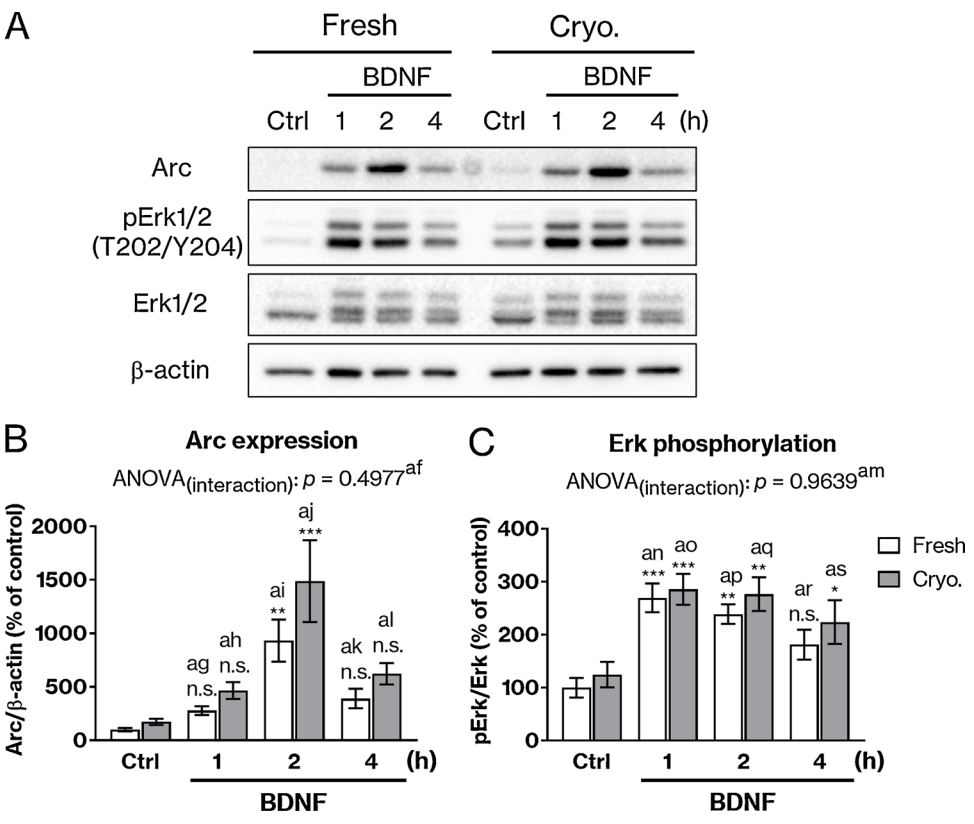


Fig. 7. BDNF reactivity was unaffected by cryopreservation. ***A,*** Typical western blots for Arc, phospho-Erk1/2 (T202/Y204), total-Erk1/2, and β-actin. The response to BDNF in freshly prepared cortical neurons and cryopreserved cortical neurons were measured. Cultures were treated with 50 ng/ml human BDNF for 1, 2, and 4 h at 21 DIV and then processed for western blotting to assess Arc expression and Erk phosphorylation. ***B,*** BDNF-induced Arc upregulation was quantified the ratio of Arc and β-actin. Data are presented as means ± SEM, $N = 6$. The interaction effect between cell type (fresh or cryopreservation) and BDNF stimulation was not detected (ANOVA, $p = 0.4977^{af}$). The effect of BDNF stimulation on Arc expression in both groups were separately analyzed by Dunnet's test for comparing among control and each BDNF stimulation groups (1, 2, and 4 h). $^{*}p < 0.05$, $^{***}p < 0.001$. ***C,*** The BDNF-induced Erk phosphorylation was quantified the ratio of phosoho-Erk1/2 and total-Erk1/2. Data are presented as means ± SEM, $N = 6$. The interaction between cell type (fresh or cryo.) and BDNF stimulation was not detected (ANOVA, $p = 0.9639^{am}$). The effect of BDNF stimulation on Erk phosphorylation in both groups were separately analyzed by Dunnet's test for comparing among control and each BDNF stimulation groups (1, 2, and 4 h). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; n.s. = non-significant.

long-term storage of rat hippocampal and cortical neurons. There is a growing need for cryopreserved neurons and consistent quality of cultured neurons is required for standardized drug safety and toxicology assessments. However, a common problem is the low viability of neurons recovered from cryopreservation. Several studies have tried to improve cryopreservation method for neuronal cells by optimizing factors such as cooling rate and thawing procedure (Das et al., 1983; Higgins et al., 2011; Quasthoff et al., 2015; Robert et al., 2016). Several companies now offer both cryopreserved neurons and freezing media. Pischedda et al. (2018) developed a new proprietary freezing media that improves the viability and retains function of cryopreserved neurons. Another study validated several commercially available freezing media and identified one commercially available reagent suitable for cryopreservation of mouse neurons (Parker et al., 2018). In these studies, many biochemical, morphological, and functional experiments were performed for assessment of cryopreserved neurons, such as gene expression, growth factor response, synapse formation, and electrophysiological properties. Also, DMSO-containing freezing media were used as a traditional freezing media in both studies. DMSO is used not only as a solvent for low soluble chemicals and drugs, but also as a cryoprotectant for freezing cells (Mandumpal et al., 2011; Yu and Quinn, 1994). In general, the components of traditional freezing media are DMSO, FBS, and culture media. However, low viability of cryopreserved neurons is one of the problems using traditional freezing media (Parker et al., 2018; Pischedda et al., 2018).

The present study shows that DMSO is suitable as a cryoprotectant for hippocampal and cortical neuronal cultures prepared by a standard protocol. Because there are many minor differences among the previous studies and this study, we cannot identify only one cause of differences. Numerous factors contribute to the quality of cryopreserved neurons: accuracy of dissection, cellular stresses in cryopreservation procedure, centrifugation, dish coating, components of media, and so on. For instance, centrifugation may have a decisive impact on the quality of cryopreserved neurons because not only centrifugation, but also repeated resuspension of cell pellet occurs excess share stress. Higgins et al. (2011) showed that centrifugation after thawing the cells significantly reduce the viability of cryopreserved neurons compared to the cells before centrifugation. Therefore, our procedures were performed without centrifugation of neurons. Furthermore, they also suggest that the process of DMSO addition and extracellular ice seeding did not cause a significant reduction in the viable cell yield. We therefore conclude that DMSO is a useful cryoprotectant for preservation of primary neurons. In addition, our method can be readily implemented as a standard laboratory practice because our cryopreserved neurons are prepared according to standard procedures without any special materials and equipment.

4.2. Cryopreserved neurons normally develop and form functional synapses

During initial neuronal development, cryopreserved hippocampal neurons showed several characteristic forms such as lamellipodia, growth cone, and future axon similar to freshly prepared neurons, with no major differences in the proportion of each stage (Fig. 2). Closer examination of stage 1 to stage 3 cells revealed that drebrin localized at the base of lamellipodia and filopodia, while F-actin was distributed to the peripheral domain of the tips of neurite. These features are consistent with previous studies (Mizui et al., 2009; Ohara et al., 2015).

The presence of functional synapses is another major criterion. The density of dendritic spines was similar between freshly prepared and cryopreserved hippocampal neurons (Fig. 3). Over the past few years, many researchers have shown interest in drebrin biology because drebrin is critical for synaptic plasticity and cognitive function (Shirao et al., 2017). Drebrin accumulation at dendritic spines is an index of mature functional synapses because the protein level of drebrin is decreased in the brain of Alzheimer's disease patients and the decreased drebrin level correlates with cognitive dysfunction (Counts et al., 2006; Harigaya et al., 1996). Intriguingly, it was revealed that drebrin loss from dendritic spines occurs prior to the loss of dendritic spines by

using amyloid β oligomers-exposed hippocampal neurons (Ishizuka et al., 2014). In addition, even in physiological conditions, drebrin translocate from dendritic spines to dendritic shafts by NMDAR stimulation: It is called drebrin exodus. This drebrin exodus is thought to be a trigger of actin-cytoskeletal remodeling during LTP (Shirao et al., 2017). Recently, drebrin came to be used as an indicator of NMDAR activity because glutamate-induced drebrin translocation is highly reproducible phenomenon (Mitsuoka et al., 2019). From the above, we adapted drebrin as a functional postsynapse marker. The densities of both synapsin I and drebrin clusters in cryopreserved neurons were comparable to freshly prepared neurons (Fig. 4A and B). Similarly, the cluster densities of other two conventional synaptic markers, VGLUT1 and PSD95 in cryopreserved neurons were comparable to freshly prepared neurons (Fig. 4C and D). We also tested the presynaptic activity of cryopreserved neurons by visualizing recycled vesicles using AM1-43 dye. The density of functional presynaptic buttons in cryopreserved hippocampal neurons was comparable to freshly prepared neurons (Fig. 4E and F). Furthermore, drebrin exodus was induced by glutamate stimulation in cryopreserved neurons same as freshly prepared neurons (Fig. 5). These results suggest that our cryopreserved neurons have functional excitatory glutamatergic synapses. We also examined the expression of pre- and postsynaptic proteins using a panel of markers for the assessment of synapse maturity. We found no significant difference in the expression of synaptic proteins between freshly prepared and cryopreserved cortical neurons (Fig. 6). Taken together, these data attest to the normal development and synapse function in the cryopreserved cultures.

4.3. Cryopreserved neurons have ability to respond to extracellular stimulation

It is important for pharmacological studies such as drug screening and toxicity assays to standardize the experimental conditions among different laboratories worldwide because sometimes the results are remarkably different among groups despite the use of similar materials and methods. Accordingly, we examined whether cryopreserved neurons respond to BDNF in the same manner as freshly prepared neurons. No statistically significant difference of interaction effect between cell type and BDNF stimulation was detected (Arc expression, $p = 0.4977^{af}$; Erk phosphorylation, $p = 0.9639^{am}$). Both Arc expression and Erk1/2 phosphorylation were temporary upregulated by BDNF-treatment in cryopreserved cortical neurons same as freshly prepared neurons (Fig. 7). In terms of cellular response, cryopreserved neurons are as competent as freshly prepared neurons.

5. Conclusion

In conclusion, our cryopreservation method for neurons brings a variety of benefits. We still have to breed to get neuronal cultures for cryopreservation. However, once we establish high-quality cryopreserved neurons, cryopreserved neurons allow us to perform experiments with primary cultured neurons that are independent from animal breeding and pregnancy or expensive commercially available neurons. In addition, banks of cryopreserved cultures allow multiple rounds of experimental testing, and the ease of transporting frozen cultures will facilitate collaboration and comparison between laboratories, and improve further standardization and quality control. Furthermore, cryopreserved neurons reduce the number of sacrificed animals in accordance to the 3Rs (Replacement, Reduction, and Refinement) and increase research productivity. We hope the method presented will help to make cryopreseved primary neuronal cultures accessible for a wide range of neuroscience applications.

Author contributions

Conceived and designed the experiments: YI. Performed the experiments: YI. Analyzed the data: YI. Wrote the manuscript: YI and CRB.

CRediT authorship contribution statement

Yuta Ishizuka: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization, Project administration. **Clive R. Bramham:** Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

None.

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