

*Short Communication***Cryopreservation of Granule Cells From the Postnatal Rat Hippocampus**Junya Ichikawa¹, Ryuji X Yamada¹, Rieko Muramatsu¹, Yuji Ikegaya¹, Norio Matsuki¹,
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Abstract. Although primary cultures of neurons are essential methods for cell biological and pharmacological researches, many animals must be sacrificed for each experiment. Here we introduce a novel system to cryopreserve hippocampal granule cells (GCs) prepared from postnatal rats. Being thawed after as long as 60 days of cryopreservation, GCs expressed the mature neuronal marker MAP-2 and elongated single tau-1-positive axons and multiple tau-1-negative dendrites. These properties closely resembled intact GCs in primary cultures, providing the advantage of being able to repeatedly prepare stable cultures with a single sacrifice of animals.

Keywords: dentate gyrus, primary culture, Prox1

A large number of animals are necessary for independent experiments to obtain cells from the same-aged animals for increasing the number of experiments. One of the methods to solve this problem is a cryopreservation of dissociated cells, which would supply cells in the same state whenever the researchers require them. Although cryopreservation of embryonic cells has been widely available (1, 2), only a few studies have succeeded in cryopreserving cells from postnatal animals (3, 4). This is probably because dissociated cells from postnatal animals require high nutrients and much oxygen, being more vulnerable to surgical procedures. In this study, we have developed a novel system to cryopreserve hippocampal granule cells (GCs) prepared from postnatal rats. The developmental states of cryopreserved GCs *in vitro* were compared with those of intact GCs in primary cultures, in terms of the survival, maturation, morphological and immunocytochemical properties, and contamination of other types of cells.

Dispersed cultures of GCs were prepared from postnatal three-day-old (P3) Wistar/ST rats (SLC, Shizuoka) as previously described (5) according to the National Institutes of Health guidelines for laboratory animal care and safety. The hippocampal formation was

dissected out from hypothermized animals. Then the subicular complex and the Ammon's horn were removed in ice-cold Gey's balanced salt solution (GBSS) enriched with *d*-glucose (6.50 g/L) and pre-aerated with 5% CO₂ and 95% air at least for 30 min. The remaining dentate gyrus was dissociated and treated with 0.25% trypsin (Nacalai Tesque, Kyoto) and 0.01% DNase I (Sigma, St. Louis, MO, USA) at 37°C for 30 min. After inhibiting trypsinization by adding horse serum, cells were centrifuged at 1200 rpm for 5 min. The supernatants were removed and remaining cells were dispersed in 2 ml Neurobasal (Neurobasal medium; Invitrogen, Gaithersburg, MD, USA) supplemented with 2% B-27 (Invitrogen) at 37°C. For primary cultures, neurons were plated onto 13-mm cover slips coated with poly-L-lysine (Sigma) at a cell density of 5.0×10^3 cells/cm² in 50% Neurobasal/B-27 and 50% astrocyte-conditioned medium at 37°C in a humidified 5% CO₂ and 95% air atmosphere. Astrocyte-conditioned medium was prepared from the cerebral cortex of neonatal Wistar/ST rats (SLC) as previously described (6). Culture medium was replaced with Neurobasal/B-27 supplemented with 2 μM cytosine-D-arabino-furanoside (Sigma) to inhibit glial proliferation, 24 and 72 h after plating. With this method, glial cells with a shape of astrocyte or microglia sparsely existed [2–3 cells per square area (see imaging methods) both in the primary cultures of intact GCs (Intact) and the cultures of cryopreserved GCs (Cryo)]

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and never reached confluence. Cultures were maintained from one to four days in vitro (DiV).

For cryopreservation, trypsinized cells were re-centrifuged at 1,000 rpm for 5 min after dispersion in Neurobasal/B-27. The supernatants were removed and cells were re-dispersed in 2 ml serum-containing Cell banker (BLC-1; ZENOAQ, Fukushima). Cell suspensions were divided into 1.2-ml freezing serum tubes (MS-4501; Sumitomo Bakelite, Akita), each of which contained approximately 10^5 cells. The freezing tubes were set in containers made of styrofoam and subsequently placed in a -80°C freezer up to 60 days. Under these conditions, cell suspensions were cooled at a rate of approximately $-1^{\circ}\text{C}/\text{min}$ (1). For experiments, cryopreserved cells were thawed rapidly by placing the freezing tube in a 37°C water bath and dispersed in 10 ml Neurobasal/B-27 at 37°C . They were centrifuged at 1000 rpm for 5 min and the supernatants were removed. Subsequently, cells were dispersed in $400\ \mu\text{l}$ Neurobasal/B-27 at 37°C and placed onto a 13-mm cover slip coated with poly-L-lysine, which was set in 24-well culture trays filled with $400\ \mu\text{l}$ astrocyte-conditioned medium per one well. They were cultured at 37°C in a humidified 5% CO_2 and 95% air atmosphere. Culture medium was replaced with Neurobasal/B-27 supplemented with $2\ \mu\text{M}$ cytosine-D-arabino-furanoside, 24 and 72 h after plating.

Cultures were incubated in 4% paraformaldehyde for 30 min at 37°C for the survival and maturation assay. For the morphology assay, they were incubated in a fixative solution consisted of 4% paraformaldehyde, $10\ \mu\text{M}$ taxol (Sigma), $1.3\ \mu\text{M}$ phalloidin (Sigma), 0.1% Triton-X, and 0.25% glutaraldehyde (Nacalai Tesque) in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl_2 , pH 6.9) for 20 min at 37°C . After washing three times with phosphate-buffered saline (PBS), samples were incubated with 2% goat serum in PBS for 1 h at room temperature and subsequently with the primary antibodies for overnight at 4°C . The samples were washed with PBS and incubated with secondary antibodies in the presence of rhodamine-conjugated phalloidin (1:40, R-415; Invitrogen), and Hoechst33342 (1:5000, H-1399; Invitrogen) for 3 h at room temperature. The primary antibodies were as follows: rabbit anti-Prox1 (1:5000, AB5475; Chemicon, Temecula, CA, USA); mouse anti-MAP-2 (1:1000, MAB378; Chemicon); and mouse anti-tau-1 (1:2000, MAB3420; Chemicon). The secondary antibodies were as follows: Alexa 594-labeled anti-rabbit IgG (1:400, A-11037; Invitrogen); Alexa 488-labeled anti-mouse IgG (1:400, A-11001; Invitrogen); and Alexa 350-labeled anti-rabbit IgG (1:400, A-11046; Invitrogen). Immunocytochemical images were acquired in both Intact and Cryo with an ORCAII cooled CCD camera (Hamamatsu

Photonics, Hamamatsu) equipped with an ECLIPSE TE300 inverted microscope (Nikon, Tokyo) and a $40\times$ objective (Nikon). Images were taken at random in at least 20 square areas ($0.69 \times 0.69\ \text{mm} = 0.48\ \text{mm}^2$) per one well and analyzed using an Aqua-Cosmos system (Hamamatsu Photonics). GCs that touched glial cells were excluded from the analysis.

To investigate the survival and development of cultured GCs, they were immunostained at DiV 4 with antibodies against Prox1, which is a specific marker of GCs (7, 8), and anti-MAP-2 antibody to detect mature neurons and were labeled with the nucleus marker Hoechst33342 (Fig. 1A). Both in Intact and Cryo, most of the MAP-2-positive neurons were also positive for Prox1 at DiV 4, and thus, GCs were the dominant cell type (Fig. 1B). The density of Prox1 and MAP-2-double-positive cells was not different between Intact and Cryo during the culture periods of DiV 1 to 4 (Fig. 1C). Only some GCs were positive for MAP-2 at DiV 1, but the vast majority of them became MAP-2-positive at DiV 4 (Fig. 1D). Overall, these data disclosed that there were no significant differences between Intact and Cryo cultures in purity, survival, and maturation ($P > 0.1$, Student's *t*-test). Furthermore, considering the strong expression of Prox1 but not of MAP-2 in the granule cell layer at P3 in vivo (Fig. 1: E–G), the progressive increase of MAP-2 in cultured GCs (Fig. 1D) was likely to reflect development rather than recovery.

Next we examined the morphology of cryopreserved GCs. Cultured cells were immunostained at DiV 4 with anti-Prox1 and anti-tau-1 antibodies, and the actin cytoskeleton was labeled with rhodamine-conjugated phalloidin (Fig. 2A). We considered the tau-1-positive neurites longer than $60\ \mu\text{m}$ as growing axons and the other tau-1-negative ones as dendrites (5, 9). Prox1-positive GCs were classified into five cell types, based on their morphology (Fig. 2B). At DiV 4, type C GCs were dominant in frequency both in Intact and Cryo cultures (Fig. 2C) and almost all Type C GCs had single tau-1-positive long neurites and multiple tau-1-negative neurites. These results indicated that cryopreserved GCs normally develop, survive, and possess axonal and dendritic morphology that are similar to those of intact GCs in primary cultures.

In this study, we demonstrated a method to prepare the culture of cryopreserved GCs of postnatal rats that was almost indistinguishable from the primary culture of intact GCs. This method provided us with the merit of being able to stock a large number of GCs from one-time sacrifice of animals. The key point of cryopreservation is obtaining healthy cells by finishing all steps before trypsinization as fast as possible (less than 90 min

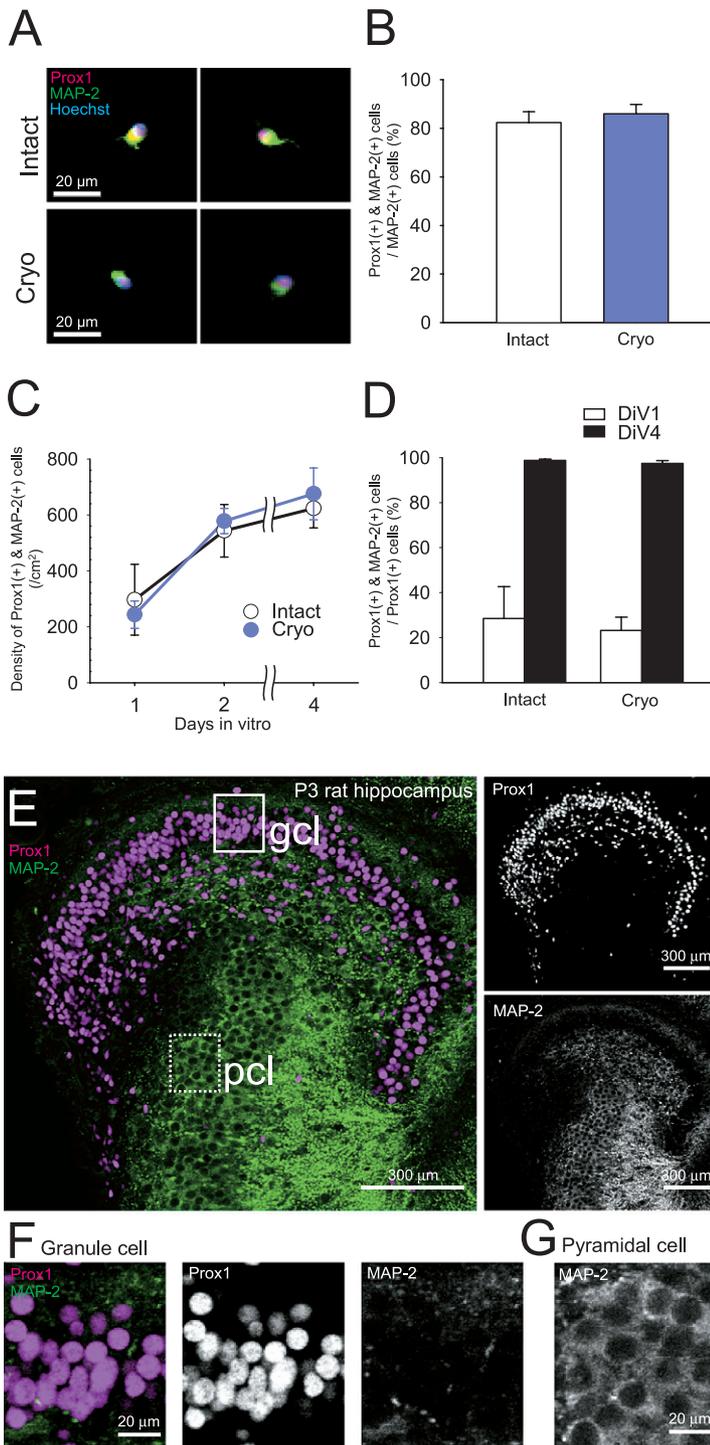
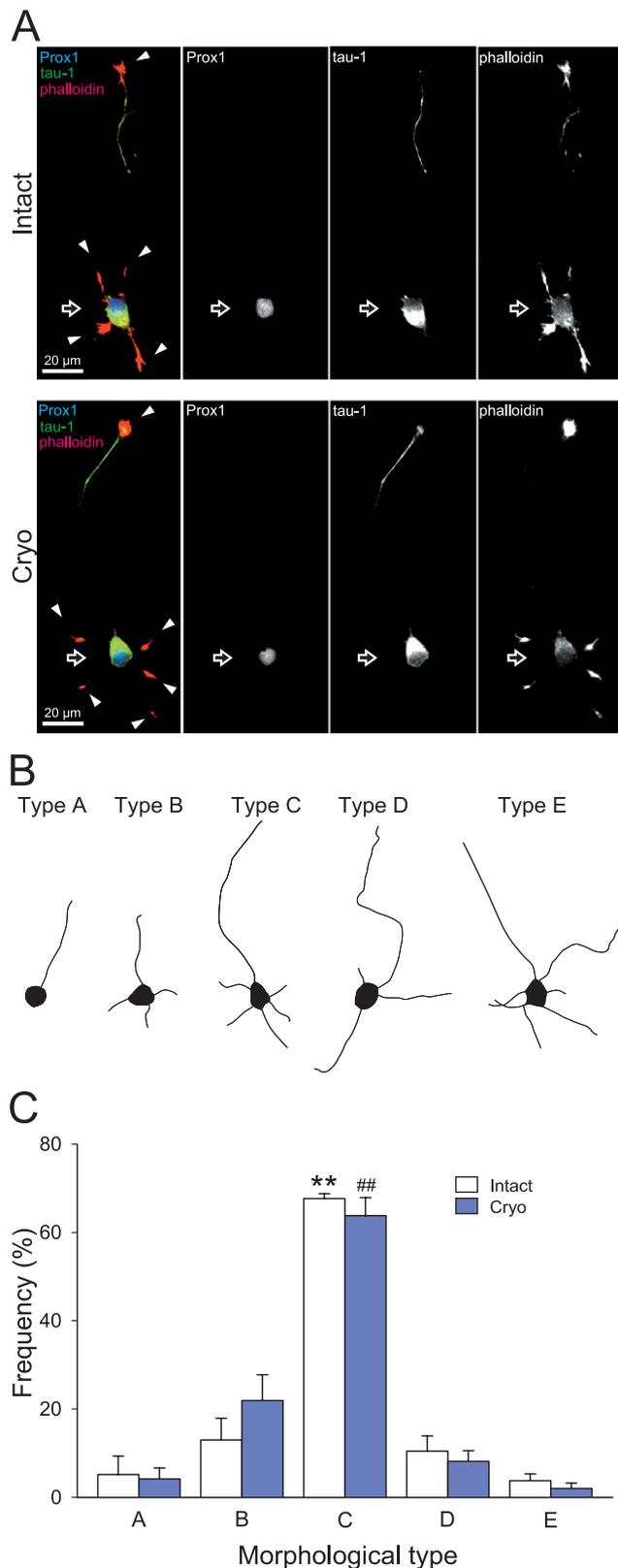


Fig. 1. The survival and development of Intact and Cryo cultures of the hippocampal granule cells (GCs). **A:** Typical fluorescent microscopic images of GCs stained with anti-Prox1 (red), anti-MAP-2 (green), and Hoechst33342 (blue) at DiV 4. **B:** The purity of Intact and Cryo cultures at DiV 4 was calculated as follows: (The number of Prox1 and MAP-2-double-positive cells / The number of MAP-2-positive cells) \times 100 (%). Most neurons (MAP-2-positive cells) in both Intact ($82.4 \pm 4.4\%$) and Cryo ($85.9 \pm 3.9\%$) cultures expressed the granule cell marker Prox1. **C:** The density (/cm²) of Prox1 and MAP-2-double-positive cells was not different between Intact and Cryo cultures during the culture periods. Considering that the density (/cm²) of Prox1-positive cells at DiV1 in Intact and Cryo were 1084.8 ± 92.5 and 1072.3 ± 50.6 , respectively, and that most of the Prox1-positive GCs did not express MAP-2 at DiV1 in both Intact and Cryo (**D**), the progressive increase of Prox1 and MAP-2-double-positive cells both in Intact and Cryo shown in (**C**) represent the maturation of immature GCs, expressing mature neuronal marker MAP-2 in a time dependent manner during culture periods. **D:** The maturation of cultured GCs was calculated as follows: (The number of Prox1 and MAP-2-double positive cells / The number of Prox1-positive cells) \times 100 (%). Although only some GCs were positive for MAP-2 at DiV 1 (Intact: $29.5 \pm 14.7\%$, Cryo: $23.2 \pm 5.8\%$), the majority of GCs became MAP-2-positive at DiV 4 (Intact: $98.8 \pm 0.7\%$, Cryo: $97.4 \pm 1.3\%$). Data are means \pm S.E.M. of each 3 cultures. **E:** A confocal image of P3 rat hippocampal slice immunostained with anti-Prox1 (magenta) and anti-MAP-2 (green) antibodies. Magnified images of the granule cell layer (solid square) and the pyramidal cell layer (dotted square) are shown in (**F**) and (**G**), respectively. Although the earliest and external marker of granule cells, Prox1, was highly expressed in the granule cell layer at this period already, almost no expression of the mature neuronal marker MAP-2 was observed. In contrast, high MAP-2 immunoreactivity was detected in the pyramidal cell layer of the same slice, confirming that the pyramidal cell layer is developmentally earlier made than the granule cell layer. Altogether, six slices from two Wistar/ST rat pups were analyzed, producing the same results. Rat pups were deeply anesthetized and perfused intracardially with PBS, followed by 4% paraformaldehyde in ice-cold PBS. Hippocampal slices (300- μ m-thickness) were prepared using a DTK-1500 vibratome (Dosaka, Kyoto). For immunohistochemistry, the samples were permeabilized for 10 min at room temperature in PBS – Triton-X (0.1%) – goat serum (5%). After permeabilization, slices were washed three times in PBS and incubated overnight at room temperature with rabbit anti-Prox1 (1:5000) and mouse anti-MAP-2 (1:1000) antibodies diluted in PBS – Triton-X (0.1%) – goat serum (5%). The slices were rinsed three times in PBS and incubated for 2 h at room temperature with Alexa 594-labeled anti-rabbit IgG (1:400) and Alexa 488-labeled anti-mouse IgG (1:400) diluted in PBS – goat serum (5%). After immunostaining, the samples were imaged with a BioRad MRC-1024 confocal system (Richmond, CA, USA) with a 20 \times objective (Nikon). Intact: the primary cultures of intact GCs, Cryo: the cultures of cryopreserved GCs, gcl: granule cell layer, pcl: pyramidal cell layer.

/10 pups). For successive cultivation of GCs from postnatal rats (both Intact and Cryo), hippocampal tissues should be treated in ice-cold and fully pre-aerated

GBSS for satisfying oxygen requirements and the astrocyte-conditioned medium should be applied during the first day in vitro to satisfy nutritional requirements.



The next step is to investigate whether this method is available for other kinds of neurons and increase the purity of cell types in cultures. Sakaguchi and colleagues have developed a system to re-cultivate dissociated cells from cultured hippocampal slices (10). The re-cultivation system allows the researchers to get specific types of cells because of the maintained topography of several cell types in the cultured slices. The application of the re-cultivation and our cryopreservation methods would provide an efficient experimental system for cell biological and pharmacological investigations.

One crucial problem that retards progress in cell-transplant or regenerative therapeutics in the central nervous system is a lack of techniques to preserve healthy neurons for long term. We chose GCs for cryopreservation in the present study because we hoped to apply the cryopreservation method to these medical treatments (11). GCs especially attract considerable attention because they are produced throughout adulthood in the dentate gyrus of mammals from rodents to humans (12). Furthermore, the axons of GCs, that is, mossy fibers, showed robust regenerative ability after axotomy in hippocampal slice cultures (6, 13–15). These neurogenetic and regenerative abilities of GCs may contribute to regenerative medicine in the central nervous system.

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Fig. 2. The axonal and dendritic growth of intact and cryopreserved granule cells (GCs). A: Typical fluorescent microscopic images of GCs of Intact (upper panels) and Cryo (lower panels) cultures at DiV 4 stained with anti-Prox-1 (blue), anti-tau-1 (green), and rhodamine-conjugated phalloidin (red). Open arrows indicate cell bodies and closed arrowheads indicate growth cones of axons and dendrites. B: Schematic drawings of cultured GCs that were morphologically classified into five cell types: Type A, a cell with a single neurite whose length was shorter than 60 μm; Type B, a cell with multiple short neurites; Type C, a cell with a single long neurite (>60 μm) that was at least three times longer than the other neurites (represented in panel A); Type D, a cell with a single long neurite (>60 μm) that was less than three times long as the other neurites; Type E, a cell with multiple neurites that were longer than 60 μm. C: At DiV 4, type C GCs were dominant in frequency and almost all Type C GCs had tau-1-positive single long neurites (Intact: 32 of 33 GCs, Cryo: 54 of 57 GCs). ** $P < 0.01$ versus Type A, B, D, E, respectively in Intact cultures and ## $P < 0.01$ versus Type A, B, D, E, respectively in Cryo cultures; Student's *t*-test. Data represent means ± S.E.M. of 49 GCs in Intact and 91 GCs in Cryo cultures. Intact: the primary cultures of intact GCs, Cryo: the cultures of cryopreserved GCs.

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