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Methods for shipping live primary cortical and hippocampal neuron cultures from postnatal mice

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ABSTRACT

Primary neuronal cultures have proven to be a powerful tool for studying mechanisms in neuroscience. It is technically challenging and expensive to reproduce high quality viable neuronal cultures. Laboratories that are not experienced or equipped to prepare primary neuron cultures may have difficulty producing consistent cultures for experiments. It has previously been shown that live rat embryonic hippocampal cultures can be shipped from laboratories that produce them. Here, we show that variations to this procedure allow for shipping postnatal mouse cultures of hippocampal and cortical primary neurons using standard commercial couriers. We also show that after shipping, primary neurons are viable, express synaptic markers, and demonstrate physiological activity, making them relevant models over immortalized cell lines. Among the many applications of this technique would be the preparation of cultured neurons from transgenic mouse lines in one laboratory and sharing them with distant collaborators, reducing variability.

1. Introduction

Primary neuron cultures have proven to be an invaluable model to study a variety of genes and pathways (White et al., 1998; Guo and Lee, 2013; Hossain and Richardson, 2020), however, in vitro models can be difficult to reproduce between collaborating labs. In some cases, studies have utilized immortalized neuron-like cells such as SH-SY5Y and HT-22 cell lines to study neuron-specific mechanisms (Liu et al., 2009; Kovalevich and Langford, 2013). Although these cells allow for controlled and reproducible experiments, they also have significant drawbacks. Immortalized cells can be quite different from brain neurons, particularly since they can proliferate, unlike neurons. Secondly, the origin of these cell lines is primarily from glioblastoma cells, which are epigenetically distant from neurons. Finally, the number of passages could allow the accumulation of substantial genetic drift (Atkins and Gartler, 1968). These properties alone limit the relevance of immortalized cell lines as a model of neuron function.

Primary neuronal cells are generally a more appropriate model for studying the CNS in vitro. Of course, even cultured neurons suffer from limitations in that synaptic network connections are disrupted during dissociation, losing anatomic specificity compared with neurons in the brain. However, while culture neurons are appropriately post-mitotic and generally capable of exhibiting spontaneous activity, their isolation and culture can be difficult, time-consuming, and expensive. Naïve lab groups may require weeks to months to learn to produce consistent cultures, making results from collaborative studies more variable. Various protocols have been established to culture primary neurons (Seibenhener and Wooten, 2012, Sahu et al., 2019). For these reasons, shipping neuron cultures between collaborating laboratories could prove to be essential in mitigating the time to produce results and ensuring better consistency.

A method published by Yang and colleagues details the process of preserving live rat hippocampal neuron cultures (Yang et al., 2010). We adapted this method for postnatal mouse cultures, further expanding the possibilities of shipping primary neurons. Central preparation of postnatal cultures allows for use of, for example, specific transgenic primary neurons to allow collaborating groups with specific technical expertise to perform complementary studies on comparable cultures, such as neurotoxicology, gene expression, physiology, and many more. By modifying this method, we have been able to routinely ship live mouse hippocampal and cortical cultures to collaborators.

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2. Methods

2.1. Animals and ethics statement

All studies complied with the ARRIVE guidelines and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Florida International University. C57BL/6J mice (Jackson Laboratory) were bred in house. Breeders were given food and water *ad libitum* and housed in a 12-h light-dark cycle. Primary neurons were dissected from postnatal C57BL/6J pups aged 0–1 days*2.2 Cell cultureware preparation*.

First, cell seeding surfaces were coated with 0.1 mg/mL Poly-L-Lysine (Sciencell, catalog # 0413, PLL) for 1 h at room temperature. PLL was removed and 5 μg/mL natural mouse laminin protein (Fisher Scientific, catalog # 23-017-015) was added and incubated overnight at 4°C. Plates can be stored up to 1 month at 4°C prior to use.

2.2. Reagent preparation

All solutions were made fresh on the day of isolation. Hippocampal and cortical tissue collection medium was prepared as 1X B-27 supplement (Fisher Scientific, catalog # A3582801) in Hibernate A (Brainbits, HA catalog # HA) and stored on ice for tissue collection. 10 mL HA was placed on ice for washing the collected tissue. Dissociation medium was prepared 2 h prior to isolations. Dissociation medium was prepared as 1 mg/mL papain (Sigma, catalog #P4762-100) in Hibernate A-calcium (Brainbits, HA-CA catalog # HACA). The enzyme solution was incubated at 37°C for 10 min to completely dissolve the papain. Once dissolved, 0.5 U/mL Dispase II (Roche, catalog # 0492078001) and 0.4 μL/mL DNAse I (Invitrogen, catalog # 18047-019) were added to the enzyme solution and kept on ice. Trituration medium was prepared as 10% Heat Inactivated Fetal Bovine Serum (Thermo Fisher, catalog # 26140079) and 1% penicillin-streptomycin (Thermo Fisher, catalog # 15140122) to in Neurobasal-A (Thermo Fisher, catalog # 10888022). Finally, culture medium was prepared by adding as 1X B-27 plus supplement, 1% L-glutamine (Fisher Scientific, catalog # 25-030-081), and 1% penicillin-streptomycin in Neurobasal-A medium. Neurons were shipped with ice cold Hibernate-E medium (Brainbits, HE catalog # HE). 0.1% Plasmocin prophylactic (Invitrogen, catalog # ant-mpp) was added to all cell culture media to prevent mycoplasma contamination.

2.2.1. Primary neuronal isolation

The hippocampi and cortices were dissected from mouse pups aged postnatal day (PND) 0–1 as previously described (Hossain and Richardson, 2020) and placed into respective collection medium tubes placed on ice (10 brains/12.5 mL medium). Once brain regions were collected, collection medium was replaced with 5 mL of HA to wash the tissue. The enzyme solution was warmed at 37°C for 5 min to allow equilibration of temperature. HA was replaced with approximately 4 mL enzyme reagent per tube. Dissected tissue in enzyme was then incubated at 37°C for 20 min. After incubation, enzyme was carefully aspirated, and dissected brains were rinsed three times with trituration medium. Dissected brains were triturated with 5 mL trituration medium using a 10 mL serological pipet. Care should be taken to not make bubbles and froth solution. After each trituration, 4 mL of free-floating cell suspension was collected and replaced with 4 mL fresh trituration medium. Trituration was repeated 3 times (twice with a 10 mL pipet and once with a 5 mL pipet). The cell suspensions were strained through a 70 μm strainer and centrifuged at 200xG for 4 min at room temperature. After centrifugation, cell pellets were observed in the tubes. The supernatant was aspirated and pellets were resuspended gently in culture medium. Cells were counted using the Cellometer Auto 2000 (Nexcelom Bioscience). While loss of cell viability during preparation of neuronal cultures has been previously reported to be approximately 20% (Seibenhener and Wooten, 2012), cell viability yields were >90% for both hippocampal and cortical primary neurons in this method as determined by trypan blue staining.

2.2.2. Seeding cells

After seeding cells, cultures were placed in an incubator at 37°C with 5% CO_2. Primary neurons were seeded at a density of 125,000 cells/cm^2. We found that this density was optimal for use in experiments such as in vitro electrophysiology after maintaining cultures for at least 14 days in vitro (DIV).

2.2.3. Shipping and maintenance of cultures

In order to ensure the viability of cultured cells, all medium changes prior to experimental testing must be half medium changes to avoid depleting nutrients and growth factors. Prior to shipping at 2 DIV, all culture medium was aspirated and immediately replaced with ice-cold HE medium filling the wells completely. Plates were sealed with an adhesive plate sealant (Fisher Scientific, catalog # AB-1170), wrapped with parafilm, covered with the lid, and once again wrapped in parafilm to ensure no spilling during shipping. Plates were placed in a Styrofoam shipping container with pre-cooled 4°C ice packs. Empty space within the container was filled with bubble wrap to minimize plate shifting. Neurons were shipped overnight from Florida International University, Miami, Florida to Rutgers University, Piscataway, New Jersey using standard UPS overnight services.

Upon arrival, neurons were unpacked and allowed to incubate in a 37°C 5% CO_2 incubator. After 2 days (4 DIV) of incubation, half of the medium was replaced with culture medium supplemented with 1X B-27 plus supplement and 5 μM cytosine β-D-arabinfuranoside (Sigma, catalog #C1768, Ara-C). Ara-C is an agent that intercalates with the DNA, inhibiting mitotic cells from proliferating, an important step in reducing glial growth in primary neuron cultures (Seibenhener and Wooten, 2012). After that, half culture medium was replaced every 3 days. For producing spontaneously active neurons, cultures were maintained until at least 14 DIV (Ichikawa et al., 1993; Biffi et al., 2013).

2.3. Cell death assay

Cell death analysis was performed with Propidium Iodide (PI; excitation/emission spectrum 535/617 nm; P4864) staining 6 h prior to shipment, and 24 h after shipment. Half of the medium was removed from each well, PI was added to a final concentration of 1 μM and incubated for 5 min. After incubation, medium containing PI was replaced with culture medium and neurons were imaged on a Nikon Diaphot microscope. Both fluorescent and phase-contrast images were taken to count nuclear PI staining. As a positive control for cell death (oxidative cell death), neurons were exposed to 200 μM H_2O_2 for 30 min prior to staining. Viability was calculated as percent of PI positive nuclei over the total number of imaged neurons.

2.4. Immunocytochemical staining

Neurons were fixed with ice cold methanol for 30 min at −20°C. Cells were then permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 10 min at room temperature. Cells were incubated in blocking buffer (3% bovine serum albumin [BSA] with 5% normal goat serum in PBS) for 1 h at room temperature. Cells were stained with microtubule associated protein 2 (Millipore Sigma, catalog # AB5543, MAP2), beta III tubulin (IIIβ-Tub), Synapsin 1 (Synaptic Systems, catalog # 106 008, Syn1), or vesicular GABA transporter (Invitrogen, catalog # MA5-24643, VGAT). Confocal imaging was performed using a Zeiss LSM700 microscope.

2.5. In-vitro electrophysiology

Whole-cell patch-clamp electrophysiology was conducted on 14 DIV cultured neurons as described (Halikere et al., 2019; Scarnati et al.,

2020). Coverslips were transferred to recording chambers with HEPES buffer consisting of (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, and 10 glucose, with the pH was adjusted to 7.4 with KOH. For both current and voltage clamp recordings a K-gluconate internal solution was used, which consisted of (in mM): 126 K-gluconate, 4 KCl, 10 HEPES, 0.05 EGTA, 4ATP-magnesium, 0.3 GTP-sodium, and 10 phosphocreatine, with the pH adjusted to 7.2 with KOH. Validity and maturity of the mouse cortical neurons was evaluated by recording spontaneous postsynaptic currents (sPSCs, at holding potential of −70 mV) and induced action potentials (at holding potential of −65 mV). Data were filtered and analyzed using Clampfit 10.5 (Molecular Devices).

2.6. Statistical analysis

Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software). All data were presented as means ± SD. Two-way ANOVA was used to assess significant differences between shipment conditions. Statistical significance was assessed as $p < 0.05$ and indicated by *.

3. Results

3.1. Characterization of mouse neurons following shipment

To confirm viability and neuronal identity of cultures following shipment, mouse primary neurons were characterized following receipt. Quality and overall maturity were evaluated after 14 DIV (12 days after shipment) using immunofluorescent staining and electrophysiology (Fig. 1A–D). Cells expressed markers of neuronal fate (IIIβ-Tub and MAP2), synaptic structures (Syn1), and GABAergic synaptic terminals (VGAT), indicating the presence of neurons interconnected with synapses. Physiological studies demonstrated that neurons in these cultures exhibited spontaneous synaptic activity after 14 DIV in culture (Fig. 1C) with a normal pattern of action potential firing following current injection (Fig. 1D). 6 h prior to shipment, cell viability was assessed by propidium iodide (PI) staining (Fig. 1E). 24 h after receipt of neurons, cell viability of shipped and non-shipped neurons was also assessed by PI staining (Fig. 1E). Analysis by Two-way ANOVA revealed that there were no significant differences in cell death between pre-shipped, non-shipped, and post-shipped primary neurons ($p > 0.05$, Fig. 1E). In all the assessed groups, treatment of 200 μM significantly increased cell death compared to each respective control ($p < 0.05$, Fig. 1E).

4. Discussion

The method described here provides for a suitable and affordable means for collaboration with laboratories that specialize in experiments evaluating primary neuron cultures. By modifying the method of Yang and colleagues, we have consistently shipped viable cultures derived from PND 0–1 mice to our collaborator over 1200 miles away. This has allowed for the opportunity to expand experimental procedures such as electrophysiology, which is currently not available in our laboratory. By utilizing this method, it will also be possible to ship neurons isolated from transgenic animals, thereby adapting in vitro models of pathology into experimental procedures. Previously, if a laboratory required primary neuronal cultures but did not have the means to isolate them, they would have to purchase them commercially from a vendor. Purchasing neurons however is very limiting since the vast majority of commercially available primary neurons are isolated from wild-type mice and are expensive. This proposed method expands on the conventional means of acquiring neural cultures from different vendors, and expands on the ability to utilize transgenic mouse lines and reduce costs.

The alternative to shipping live cultures is to prepare and ship cryopreserved neurons. There have been multiple methods published detailing a variety of cryopreservation techniques of primary neurons (Higgins et al., 2011, Quasthoff et al., 2015, Robert et al., 2016; Pischedda et al., 2018; Ishizuka and Bramham, 2020). While these

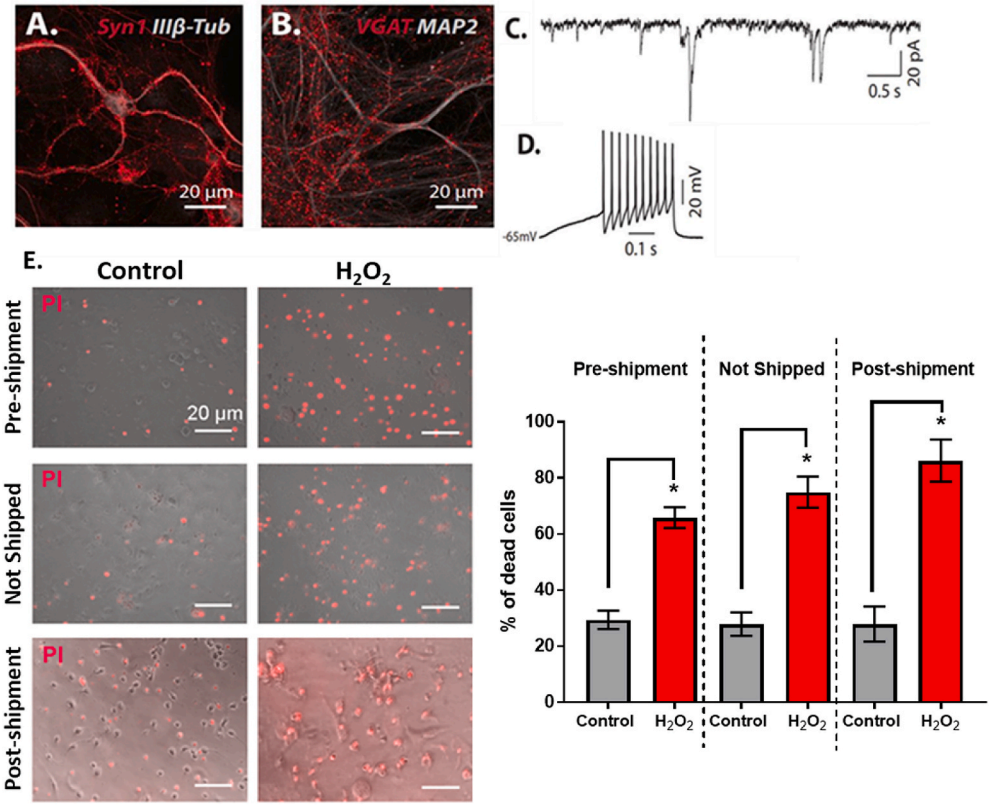

Fig. 1. Identity and viability of mouse neurons after shipment. A. Expression of neuron-specific IIIβ-tubulin (IIIβ-Tub) and the synaptic markers synapsin 1 (Syn1) in 14 DIV neurons following a shipment. B. Expression of IIIβ-Tub and a marker of GABAergic synaptic terminals (VGAT, vesicular GABA transporter) in 14 DIV neurons. C. Spontaneous post synaptic currents (sPSCs) recorded from 14 DIV mouse neurons. D. Induced action potential firing of 14 DIV neurons. E. PI staining of mouse neurons prior to shipment (2 DIV), after shipment (4 DIV), and non-shipped conditions (4 DIV). All data are presented as means ± SD. Significant differences to control are represented as *, $p < 0.05$, as determined by Two-way ANOVA. N = 6.

methods may serve as potentially effective solutions, they require optimization of additional steps and longer recovery times than the direct shipment of live cultures. Additionally, these methods allow for longer term storage than shipping of live primary neurons, however, the vast majority of these publications focus on the viability of the neuronal cultures after thawing for various times (Higgins et al., 2011, Robert et al., 2016; Ishizuka and Bramham, 2020). It is important to note that the viability of cultures after cryopreservation has been observed to be quite variable between different methods and freezing times. While viability is obviously important, it is also important to investigate the maturation of these cultures when compared to fresh live cultures, which we have shown here with electrophysiology experiments. While this technique is favorable for long term storage of cultures, the method was only shown utilizing embryonic mouse pups for dissections. Additionally, this method requires materials and reagents which are not commercially available and have a proprietary composition (i.e., NeuroStore) (Pischedda et al., 2018). All reagents and materials used in the method described here are commercially available.

Another strategy is to ship cryopreserved tissue to prepare primary cultures in the receiving laboratory. This method has been shown to be effective for laboratories that do not have access to specific transgenic mouse models. There is evidence that neurons isolated from cryopreserved tissue also retain functional neuronal properties (Cano-Jaimez et al., 2020). However, this method requires that the receiving laboratory has the experience and personnel to efficiently isolate neurons from tissue. Furthermore, it has been shown that the yield of viable neurons decreases significantly after cryopreservation of the neural tissue (Cano-Jaimez et al., 2020).

We have shown that shipped cultures of primary mouse neurons are viable and continue maturation, producing spontaneous activity. These findings are important to establish that no confounding factors arise from cell death or maturation abnormalities that could further impact the experiments. Furthermore, we show that mature synaptic markers are present in shipped cultures at 14 DIV. Since neurons are shipped early in culture (2 DIV), this also expands the range of experimental techniques available using this model, such as studies focusing on the development of synapses throughout culture. We have not explored the potential ability of shipping primary neurons past 2 DIV. However, previous reports have shown that neuronal health decreases as the age of neurons at shipping increases (Yang et al., 2010).

To our knowledge, there are no reports that detail the shipment of live primary mouse neurons, extracted from postnatal pups, demonstrating normal activity as shown by electrophysiology. By using live models, laboratories circumvent the possibility of unknown factors associated with the cryopreservation of neuronal cells or tissue, and deliver viable and functional cultures. The utilization of primary mouse neurons for shipment expands the capabilities of in vitro studies to include transgenic models highly characterized in pathological models of disease, as well as reduce the burden associated with embryonic dissections. Additionally, deriving primary neurons from postnatal mice reduces the animal burdens associated with embryonic dissections. By utilizing the methods described here, laboratories without the capability and access to culturing primary neurons will be able to collaborate and become independent of expensive commercial primary cells. Furthermore, based on these methods, we have also successfully shipped human iPSC-derived neurons between laboratories with similar success (results not shown). This simple method will effectively lower the cost and burden of obtaining quality primary neuronal cultures.

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Ethical statement

The animal study protocol was approved by the Institutional Animal Care and Use Committee of Florida International University (approval code: IACUC-21-047). All mice were housed at Florida International University Animal Care Facility. Procedures were conducted in accordance with the National Institutes of Health's Guide for Care and Use of Laboratory Animals.

CRediT authorship contribution statement

Ferass M. Sammoura: Conceptualization, Methodology, Writing – original draft, Investigation, Visualization. **Dina Popova:** Methodology, Writing – original draft, Validation, Investigation, Visualization. **Ayeshia Morris:** Investigation. **Ronald P. Hart:** Writing – review & editing, Supervision. **Jason R. Richardson:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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The graphical abstract was created on Biorender.com.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crneur.2022.100069.

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