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A rapid and efficient method for dissociated cultures of mouse myenteric neurons

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HIGHLIGHTS

- Myenteric neurons can be isolated from multiple mice in under 2 h.
- A high yield of neurons and few non-neuronal cell types are present.
- Many neuronal subtypes are present, reflecting the in vivo population.
- Neurons are optimal at 3 weeks but can survive for at least 5 weeks.

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ABSTRACT

Background: The enteric nervous system controls gastrointestinal functions such as secretion and smooth muscle contraction/relaxation. Neuronal enteric dysfunction is a feature of many direct gastrointestinal disorders and can be secondary to central nervous system disorders. Research in this field has been limited and there are few published methods on dissociated enteric cultures.
New method: Here we describe a quick and efficient method for culturing myenteric neurons which optimizes neuronal yield. A simplified technique is presented to easily dissect the myenteric plexus and longitudinal muscle from the outside of the intestinal wall reducing non-neuronal cell and bacterial contamination from the final culture. These segments are subjected to enzymatic dissociation and the resulting neurons are placed into an optimal growth media for long term culture.
Results: This protocol produces a high yield of neuronal cells. Multiple neuronal subtypes reflecting the in vivo population are observed. Cultures are optimal at 3 weeks in vitro but can be sustained for at least 5 weeks.
Comparison with existing methods: Unlike other protocols our method does not require a time consuming challenging dissection, long enzymatic treatment times or the use of specialized equipment. Resulting cultures are of higher quality and can be sustained longer permitting proper neuronal recovery. In addition cell attachment to culture substrates have been optimized.
Conclusion: We provide a novel method for researchers to dissociate and grow high quality enteric neuronal cultures. Our method can be used for studies on gastrointestinal diseases caused by enteric neuronal dysfunction and to explore possible pharmacological interventions in vitro.

Abbreviations: ChAT, choline acetyltransferase; CM, circular muscle; CNS, central nervous system; DAPI, 4′-6-diamidino-2-phenylindole; DMEM/F12, Dulbecco's modified eagle medium: nutrient mixture F-12; ENS, enteric nervous system; GFAP, glial fibrillary acidic protein; GI, gastrointestinal; HBSS, Hank's balanced salt solution; ICC, interstitial cell of Cajal; LM, longitudinal muscle; LMMP, longitudinal muscle [plus] myenteric plexus; MG, matrigel; MP, myenteric plexus; NeuN/Fox-3, neuronal nuclei; nNOS, neuronal nitric oxide synthase; PBS, phosphate buffered saline; PDL, poly-D-lysine; SMP, submucosal plexus; Tuj-1, neuron-specific class III beta-tubulin; VIP, vasoactive inhibitory peptide.

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1. Introduction

The enteric nervous system (ENS) regulates gastrointestinal (GI) tract activity with a degree of executive input from the central nervous system (CNS). The ENS consists of two main layers. The submucosal plexus (SMP) which is abluminal to the mucosa and the larger myenteric plexus (MP) layer which is located between the circular muscle (CM) and the longitudinal muscle (LM). These neuronal plexuses exert control over secretion and absorption, bidirectional fluid movement, GI blood flow, and control smooth muscle contraction and relaxation related to peristalsis (Furness, 2008).

Immunohistochemical and electrophysiological studies of the ENS are critical to the characterization of the pathophysiology of enteric disorders. For example, Hirschsprung disease results from incomplete migration of neurons comprising the distal ENS, starting at a variable length proximally and extending to the rectum (Amiel et al., 2008). In addition to Hirschsprung disease, diabetic neuropathy, inflammatory bowel diseases, ischemia and appendicitis can also result in ENS neuropathology (Bagyánszki and Bódi, 2012; Villanacci et al., 2008; Piao et al., 1999; Xiong et al., 2000). It is increasingly clear that the ENS can also be affected in diseases thought to be primarily central neurological disorders. These include Parkinson's disease and Alzheimer's disease (Derkinderen et al., 2011; Semar et al., 2012). There is also evidence that the ENS can be affected in neurodevelopmental conditions such as Rett syndrome and autism spectrum disorders resulting in clinical gastrointestinal complications as a comorbidity (Wahba et al., 2015; Frye et al., 2015). Tissue culture studies are often a useful adjunct to probe the pathophysiology in animal models of these disorders.

In a literature survey only very few enteric neuronal dissociation tissue culture protocols are found. We determined that the methods currently available (Saffrey et al., 1991; Jaeger, 1995; Smith et al., 2013; Zhang and Hu, 2013; Grundmann et al., 2015) are complex, time consuming, expensive and/or equipment intensive. A simpler, faster method will likely lead to wider application of tissue culture studies. We describe a technique for dissociation of enteric neurons which produces abundant, healthy cultures within 2–3 h. These cultures remain robust for at least 3 weeks and survive in culture for at least 5 weeks.

2. Materials and methods

2.1. Enteric neuronal dissociation

2.1.1. Harvesting of GI tract

CD-1 mice aged 4–6 months were anesthetized with isoflurane and euthanized by cervical dislocation. The abdomen was sterilized with 70% ethanol. An incision was made from the end of the sternum to the pelvis to open the abdominal cavity and expose the GI tract (Fig. 1A and B). The cecum was identified, in the lower abdomen, and the small intestine was removed immediately distal to the stomach and ending at the cecum (Fig. 1C and D). The small intestine was cut into approximately 4 cm pieces and placed in ice cold Krebs-Ringer solution (126 mM NaCl, 5 mM KCl, 1.25 mM Na_2HPO_4, 1.25 mM $MgCl_2$, 2.5 mM $CaCl_2$, 11 mM glucose, 25 mM $NaHCO_3$) stored on ice. A curved blunt ended 20 gauge feeding needle (Fine Science Tools) attached to a 25 mL syringe containing ice cold Krebs-Ringer solution was inserted into the lumen of each intestinal segment to remove and rinse out the intestine of all contents (chyme; Fig. 1E). After washing out the contents, each intestinal segment was placed into ice cold Krebs-Ringer solution on ice until further processing.

2.1.2. Separation of MP

Individual intestinal segments were slightly stretched and pinned at each corner of the segment on a silicone (Sylgard 184; Dow Corning) coated 10 cm plate containing pre-chilled Krebs-Ringer solution (Fig. 1F). The longitudinal muscle and adherent myenteric plexus (LMMP), the external layer, was separated from the other gut layers beginning at one end of the segment on the serosal surface. Gentle stroking abrasion was provided using the convex face of a pair of curved extra fine forceps (Excelta 7-SA) until the LMMP pulled away from the remaining tissue (Fig. 1G). This was continued for the length of the segment to cause separation of the LMMP from the circular muscle (Fig. 1H & Video 1). The remaining GI tissue lacking the LMMP was discarded. All isolated LMMP segments were stored together in ice cold Krebs-Ringer solution in a 15 mL conical tube on ice until all segments were dissected.

2.1.3. Enzymatic digestion/dissociation

After isolation of all LMMP strips, the 15 mL conical tube containing the Krebs-Ringer solution and LMMP strips was centrifuged at 200 g for 5 min and the supernatant was aspirated. The tissue was resuspended in pre-warmed (37 °C) collagenase digestion solution (2 mL of solution per mouse; 1 mg/mL collagenase IV, 0.5 mM $CaCl_2$, 10 mM HEPES in Hank's balanced salt solution (HBSS)). Strips were incubated in this collagenase solution in a 37 °C water bath for 15 min with manual gentle constant rotation of the tube plus inverting the tube once every 5 min. The myenteric ganglia do not utilize collagen for tissue adhesion and therefore the collagenase treatment dissociates and removes muscular tissue from the myenteric plexus (Gershon and Rothman, 1991). Following this, the tissue was centrifuged at 200 g for 5 min and the supernatant was aspirated. The resulting pellet was washed 2 times with 10 mL ice-cold HBSS (Sigma Aldrich) for 5 min with constant gentle inversion. After washing, the tissue was left to settle for 3 min and the supernatant was removed. Next, pre-warmed (37 °C) trypsin digestion solution was added to the tissue (1 mL of solution per mouse; 0.05% Trypsin, 0.53 mM EDTA in HBSS) and incubated for 10 min in a 37 °C water bath for 15 min with manual gentle constant rotation of the tube plus inverting the tube once every 5 min. The trypsin breaks up and releases individual neurons from the ganglia. Following this the cells were centrifuged at 200 g for 5 min and the supernatant was removed. The pellet was triturated 20× in culture media (2% (v/v) fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 1× B27 supplement, 1× N2 supplement, 7.2 mg/L uridine triphosphate, 15.6 mg/L 5-flurodeoxyuridine, 2.5 mg/L amphocetrin, 50 mg/L gentamycin sulfate in Dulbecco's modified eagle medium: nutrient mixture F-12 (DMEM/F12) media) with a 1 mL pipette, debris was left to settle for 1 min and the supernatant was transferred to a 15 mL conical tube. Cells were diluted to the volume required for plating (see below). Cells are not evident at this stage due to remaining debris that will be removed with serial media changes during cell culture. This makes accurate cell counts at plating impossible. Exact plating volumes should be titrated and based on desired density, mouse number and mouse size. We recommend the resulting cells from one adult mouse be used for 5–8 wells of a 96 well culture dish.

2.1.4. Plating enteric neurons

After the plates were treated with the indicated culture substrate (see below), all wells were washed with pre-warmed (37 °C) HBSS once and cells were then plated onto the wells. Depending on desired culture density, 5–8 wells per mouse on a 96-well dish can be plated (200 μL per well) on pre-coated wells. After plating, cells were grown in an incubator at 37 °C with 5% CO_2. A complete media change was performed every day; the media was suctioned using a