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Characterization of a novel primary culture system of adult zebrafish brainstem cells

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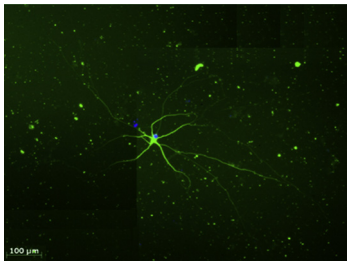
HIGHLIGHTS

- A method for culturing adult zebrafish primary brainstem cells is described.
- Cultures contain a majority of neurons, with smaller populations of glial cells and stem progenitor cells.
- The culture presents a novel tool to investigate CNS regeneration mechanisms.

GRAPHICAL ABSTRACT



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ABSTRACT

Adult zebrafish (*Danio rerio*) have a remarkable ability to restore function after an injury to the brain or spinal cord. The molecular and cellular mechanisms underlying this phenomenon are not fully understood. To enable investigation of these mechanisms we have developed an *in vitro* model system from the adult zebrafish brainstem, which can be maintained under serum-containing and serum-free conditions. While cultures are predominantly neuronal, they also contain glia and stem progenitor cells. Various stages of cellular differentiation are observed among both neuronal and non-neuronal populations. Quantitative morphological results revealed typical cellular growth over a two-week period. We argue that our novel brainstem culture model offers a powerful tool for the studies of axonal growth, neurogenesis, and regeneration in the adult zebrafish central nervous system.

Abbreviations: ANOVA, analysis of variance; BrdU, 5-bromo-2′-deoxyuridine; CNS, central nervous system; *div*, days *in vitro*; DNase, deoxyribonuclease; EDTA, ethylenediaminetetraacetic acid; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; HuC/D, human neuronal protein C/D; MAb, monoclonal antibody; PAb, polyclonal antibody; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; SEM, standard error of the mean; Tph2, tryptophan hydroxylase 2.

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1. Introduction

The adult teleost zebrafish, *Danio rerio*, recovers motor (swimming) function after central nervous system (CNS) trauma (reviewed in Bernhardt, 1999). It has been demonstrated that brainstem neurons, which project to the spinal cord, regenerate axons after spinal cord injury (Becker et al., 1997). Studies have also shown that neurogenesis is induced by spinal cord and traumatic brain injury (Reimer et al., 2008; Kizil et al., 2012a). Both of these processes, axon regeneration and neurogenesis, contribute to anatomical and functional recovery in the injured CNS of adult zebrafish. Its intrinsic growth and repair capacity, combined with its experimental amenability, presents the zebrafish as a potent model for investigations of CNS restoration (Sperry, 1947; Skromne and Prince, 2008).

Previous studies have yielded insight into the roles of stem/progenitor cells and the immune system in successful zebrafish regenerative/repair programs (Kroehne et al., 2011; Kyritsis et al., 2012). In addition, a few genes involved in axonal regeneration in the mature zebrafish CNS have been identified (reviewed in Guo et al., 2011; Kizil et al., 2012b). However, at present the cellular and molecular mechanisms fundamental to the restorative capacity of zebrafish are still incompletely understood (reviewed in Vajn et al., 2013). *In vitro* model systems of adult neural cells are vital for the investigation of the role of molecules and cells in CNS repair because they offer controlled conditions allowing precise molecular manipulations.

Some *in vitro* model systems have demonstrated their potential for studies on CNS injury and repair (Becker and Becker, 2002; Sakowski et al., 2012). However, so far a model system of adult zebrafish primary brain neurons has not been described. Here, we report on an *in vitro* model system of neural cells from adult zebrafish brainstem. This region of the brain contains neurons that project to the spinal cord and control swimming activity. We present morphological data and a detailed cellular characterization supporting the use of this novel *in vitro* tool in investigations of axonal growth, neurogenesis, and regeneration in the damaged CNS.

2. Materials and methods

2.1. Zebrafish

The adult (>3 months old), wild-type, male and female zebrafish used in our studies were obtained from a local fish breeder. All zebrafish-related procedures were approved by the St. Thomas University Institutional Animal Care and Use Committee.

2.2. Adult primary brainstem cell culture

Zebrafish were deeply anesthetized in 0.033% aminobenzoic acid ethylmethylester (Tricaine; Sigma, St. Louis, MO, USA) in normal aquarium water and then decapitated. Brains were dissected and rinsed three times in sterile phosphate buffered saline (PBS; pH 7.4). The brainstem (Fig. 1A) was removed and incubated in trypsin (0.25% trypsin–ethylenediaminetetraacetic acid (EDTA; Invitrogen, Grand Island, NY, USA) at 28.5 °C for five minutes. Complete L-15 medium [Leibovitz-15 medium (Invitrogen), 2% fetal bovine serum (FBS) (Invitrogen), 100 U/mL penicillin-100 μg/mL streptomycin (Sigma), and 1× N_2 supplement (Invitrogen)] with 100 mM $CaCl_2$ was added to the trypsinized tissue. Supernatant was drawn off and replaced with fresh Complete L-15 medium, $CaCl_2$, and DNAse I (5 mg/mL; Sigma). Brainstems were triturated fifty times with a fire-polished large bore pipette and then five times with a fire polished small bore pipette. Complete L-15 medium and $CaCl_2$ were added to the triturated tissue and centrifuged at 80 × *g* for five minutes. Supernatant was discarded, and pelleted cells were resuspended in complete L-15 medium. Cells were seeded at a density of two brainstems (approximately 300,000 cells) per 4.2 cm^2 tissue culture well. Cells were grown at 28.5 °C with only atmospheric CO_2 on Nunc Permanox two-well chamber slides (Thermo Scientific Lab-Tek; Rochester, NY, USA). Prior to cell seeding, these slides were coated with Protran nitrocellulose (Whatman, GE Healthcare, Piscataway, NJ, USA), dried, sterilized under UV light for 15 min, coated with poly-D-lysine (0.5 mg/mL; Sigma) for at least four hours, rinsed with sterile water, coated with laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane (1.7 μg/mL; Sigma) overnight at 4 °C (Lagenaur and Lemmon, 1987; Dou and Levine, 1994; Becker and Becker, 2002), and finally rinsed four times with PBS. Cells were grown for one, three, seven, or fourteen days with medium changes at day two and, in the case of fourteen-day cultures, at day nine.

2.3. Establishment of serum-free culture conditions

For serum-free cultures, the dissociated brainstem cells were first seeded in Complete L-15 medium (see above), which was substituted with FBS-free Complete L-15 medium at day two. The cells were grown for a total of three, seven, or fourteen days with an additional medium change at day nine in the case of fourteen-day cultures.

2.4. Bromodeoxyuridine labeling

To monitor cell proliferation *in vitro*, dissociated brainstem cells were seeded in Complete L-15 medium containing 500 μM 5-bromo-2′-deoxyuridine (BrdU; Sigma) and cultured for one or seven days. BrdU containing media was exchanged with Complete L-15 media at day two.

2.5. Immunocytochemistry

Cultures were fixed in 4% paraformaldehyde with 4% sucrose at room temperature for 20 min, rinsed with PBS, and incubated in blocking solution [PBS, 1.5% normal goat serum (Invitrogen), and 0.3% Triton X-100 (Fisher Scientific, Pittsburgh, PA, USA)] for 30 min. Cultures were incubated with different antibodies raised against tubulin: 6G7 [monoclonal antibody (MAb), mouse, 1:250; Developmental Studies Hybridoma Bank (DSHB), Iowa City, Iowa, USA]; SAB2102604 [polyclonal antibody (PAb), rabbit, 1:250; Sigma]; CBL270 (MAb, rat, 1:250; EMD Millipore, Billerica, MA, USA) recognizes alpha-tubulin; and T6793 (MAb, mouse, 1:1000; Sigma) labels acetylated tubulin. Acetylated tubulin is routinely used to label zebrafish neuron axon tracts *in vivo* (Wilson et al., 1990; Kim et al., 2008). 6G7 and SAB2102604 label the same cells as T6793 in our cultures and CBL270 labels T6793 positive cells, as well as additional flat cells with glia morphology (data not shown). Cultures were also immunostained with A-21271 (MAb, mouse, 1:500; Molecular Probes-Invitrogen) for HuC/D detection; Zrf-1 [MAb, mouse, 1:500, Zebrafish International Resource Center (ZIRC), Eugene, OR, USA] to detect glial-fibrillary acidic protein (GFAP); 40E-C (MAb, mouse, 1:50; DSHB) to recognize vimentin; 2E5 (MAb, rat, 1:2, kindly donated by Dr. Laure Bally-Cuif, Zebrafish Neurogenetics Group, Institute of Neurobiology Alfred Fessard, Gif-sur-Yvette cédex, France) to recognize tryptophan hydroxylase 2 (Tph2) utilizing a blocking solution containing 0.1% Tween 20 (Carolina Biological Supply Company, Burlington, North Carolina, USA), 1% bovine serum albumin (Promega, Madison, Wisconsin, USA) and 1.5% normal goat serum in PBS; and 55858P (PAb, rabbit, 1:500; AnaSpec, Fremont, CA, USA) to detect nestin. The specificity of the nestin antibody was confirmed in adult zebrafish brain