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Neuronal development and migration in zebrafish hindbrain explants

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Abstract

The zebrafish embryo is an excellent system for studying dynamic processes such as cell migration during vertebrate development. Dynamic analysis of neuronal migration in the zebrafish hindbrain has been hampered by morphogenetic movements in vivo, and by the impermeability of embryos. We have applied a recently reported technique of embryo explant culture to the analysis of neuronal development and migration in the zebrafish hindbrain. We show that hindbrain explants prepared at the somitogenesis stage undergo normal morphogenesis for at least 14 h in culture. Importantly, several aspects of hindbrain development such as patterning, neurogenesis, axon guidance, and neuronal migration are largely unaffected, inspite of increased cell death in explanted tissue. These results suggest that hindbrain explant culture can be employed effectively in zebrafish to analyze neuronal migration and other dynamic processes using pharmacological and imaging techniques.

Keywords: Zebrafish hindbrain; Explant culture; Rhombomere; Motor neuron; Neuronal migration; Time-lapse microscopy; Green fluorescent protein

1. Introduction

The transparent zebrafish embryo is an established model system for studying vertebrate development. The zebrafish is genetically manipulable, leading to the identification, by microscopic observation of live embryos, of hundreds of mutations affecting embryonic patterning and morphogenesis (Haffter et al., 1996; Driever et al., 1996). Moreover, the transparency of the embryo has facilitated the observation and analysis of dynamic cellular processes during epiboly and gastrulation (Warga and Kimmel, 1990; Concha and Adams, 1998; Jessen et al., 2002), hematopoieisis (Herbomel et al., 1999), neuronal migration (Koster and Fraser, 2001; Jessen et al., 2002), and growth cone guidance (Eisen et al., 1986; Hutson and Chien, 2002; Gilmour et al., 2002).

Despite the obvious advantages of observing dynamic processes in the intact embryo, there are experimental limitations to this approach. First, most studies thus far have analyzed dynamic cellular processes occurring in superficial embryonic tissues that can be imaged at high resolution, while deeper tissues especially in the head have been less accessible due to the curvature of the head around the yolk cell. Second, immobilization of intact embryos for long-term observations (3 h or longer) hinders normal morphogenetic movements, potentially affecting the process under observation. Finally, the embryonic skin is tough and quite impermeable, potentially precluding the use of many pharmacological reagents that have been used very effectively to study dynamic processes in cultured cells.

To overcome these problems, Langenberg et al. (2003) devised a culture system that supports growth and development of a deyolked zebrafish embryo over long time periods. Furthermore, this culture system was conducive to the observation of dynamic processes in explants of the zebrafish nervous system (Langenberg et al., 2003). However, before the explant culture technique can be used for studying particular cell types, one must rigorously test whether the explanted tissue in the region of interest preserves the native (endogenous)

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environment, and whether the cell type to be studied behaves normally in explant culture. We are investigating the cellular and molecular mechanisms underlying facial branchiomotor neuron (FBMN) migration in the zebrafish hindbrain (Bingham et al., 2002; Jessen et al., 2002; Chandrasekhar, 2004). FBMN migration has been intensively studied in mouse (Garel et al., 2000; Studer, 2001) and zebrafish (our work; Cooper et al., 2003) as a model for tangential neuronal migrations in the vertebrate brain (Marin and Rubenstein, 2001). Therefore, we investigated whether zebrafish explant culture could be another tool in our studies of FBMN migration. We show here that tangential (caudal) migration of FBMNs occurs normally in cultured hindbrain explants. The explants remain healthy for at least 14 h in culture, and several aspects of hindbrain development and patterning are mostly unaffected. These observations indicate that the analysis of FBMN migration in hindbrain explants is a powerful complementary approach to the analysis of this dynamic process in the intact zebrafish embryo.

2. Materials and methods

2.1. Animals

Maintenance of zebrafish stocks, and collection and development of embryos in E3 embryo medium were carried out as described previously (Westerfield, 1995; Chandrasekhar et al., 1997; Bingham et al., 2002). To facilitate analysis of branchiomotor neuron development, fish carrying the motor neuron-expressed *islet1-GFP* transgene (Higashijima et al., 2000) were used to obtain embryos for all experiments. Throughout the text, the developmental age of the embryos corresponds to the hours elapsed since fertilization (hours post fertilization, hpf, at 28.5 °C).

2.2. Explant preparation and mounting

The procedures for removing the yolk cell, generating explants, and mounting for observation were essentially the same as those described previously (Langenberg et al., 2003; Fig. 1), with the following modifications. The non-hydrolyzable ATP analog (AMP-PNP, Sigma) was prepared to a concentration of 50 mM by dissolving in 100 mM phosphate buffer (pH 7.4) containing 0.1% phenol red. The stock L-15 amphibian culture medium (GibcoBRL) was diluted to 67% with sterile water, and supplemented with tissue culture penicillin/streptomycin cocktail (1× final), and 1 M glucose (10 mM final). Following AMP-PNP injection, embryos were deyolked in sterile E3, washed twice in E3, transferred and maintained in L-15 solution (80% stock L-15, 20% sterile E3) until all explants were ready for extended incubation. Hindbrain explants were obtained by cutting deyolked embryos with a fine scissors in the rostral spinal cord. For long-term culture, up to eight explants were transferred under sterile conditions to a well in 24-well tissue culture plates containing

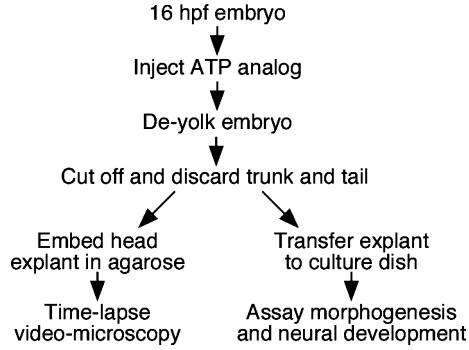

Fig. 1. Generation and analysis of hindbrain explants. Embryos were injected with the ATP analog (AMP-PNP) in the yolk cell. Following yolk removal, the embryo was decapitated, and the head fragment containing the hindbrain was either embedded in agarose for imaging and video microscopy or transferred to culture medium for assaying morphogenesis and neural development at subsequent stages. (see Section 2 for details).

1 ml/well 67% L-15 medium (see above). Control embryos were dechorionated and incubated in identical conditions to explants.

Embryos or hindbrain explants were embedded in 0.4% agarose for time-lapse microscopy. Briefly, an explant or embryo was transferred to a 1.5 ml microfuge tube containing 200 μl of 100% L-15 medium, supplemented with Penicillin/Streptomycin cocktail and glucose (but no water). To this tube, 100 μl of melted agarose solution (1.2% agarose dissolved in sterile water, and maintained at 55 °C) was added, mixed gently, and the tissue was manipulated into the desired orientation in a small drop of agarose/L-15 solution placed on a pre-warmed microscope slide. Upon gelling, the agarose above the area of interest was gently removed, the agarose drop was covered with 67% L-15 solution, and the explant/embryo was observed using long-working distance objectives on an Olympus BX60 microscope. Bodipy ceramide labeling (to assay neuroepithelial cell shapes) and acridine orange labeling (to assay cell death) of explants and embryos were performed essentially as described (Brand et al., 1996; Cooper et al., 1999), and the tissue was embedded as described above. Acridine orange-labeled tissue was imaged using epifluorescence on the BX60 microscope, and bodipy ceramide-labeled tissue was imaged on an Olympus IX70 microscope equipped with a BioRad Radiance 2000 confocal laser system.

2.3. Immunohistochemistry, in situ hybridization, and imaging

Whole-mount immunohistochemistry was performed with various antibodies as described previously (Chandrasekhar et al., 1997; Bingham et al., 2002; Vanderlaan et al., 2005). Synthesis of the digoxygenin- and fluorescein-labeled probes, and whole-mount in situ hybridization were carried out as described previously (Chandrasekhar et al., 1997; Prince et al., 1998; Bingham et