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ScienceDirect
Developmental Biology 313 (2008) 167–180
DEVELOPMENTAL BIOLOGY
www.elsevier.com/developmentalbiology

The zebrafish cerebellar rhombic lip is spatially patterned in producing granule cell populations of different functional compartments

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Received for publication 24 April 2007; revised 18 September 2007; accepted 15 October 2007
Available online 24 October 2007

Abstract

The upper rhombic lip, a prominent germinal zone of the cerebellum, was recently demonstrated to generate different neuronal cell types over time from spatial subdomains. We have characterized the differentiation of the upper rhombic lip derived granule cell population in stable GFP-transgenic zebrafish in the context of zebrafish cerebellar morphogenesis. Time-lapse analysis followed by individual granule cell tracing demonstrates that the zebrafish upper rhombic lip is spatially patterned along its mediolateral axis producing different granule cell populations simultaneously. Time-lapse recordings of parallel fiber projections and retrograde labeling reveal that spatial patterning within the rhombic lip corresponds to granule cells of two different functional compartments of the mature cerebellum: the eminentia granularis and the corpus cerebelli. These cerebellar compartments in teleosts correspond to the mammalian vestibulocerebellar and non-vestibulocerebellar system serving balance and locomotion control, respectively. Given the high conservation of cerebellar development in vertebrates, spatial partitioning of the mammalian granule cell population and their corresponding earlier-produced deep nuclei by patterning within the rhombic lip may also delineate distinct functional compartments of the cerebellum. Thus, our findings offer an explanation for how specific functional cerebellar circuitries are laid down by spatio-temporal patterning of cerebellar germinal zones during early brain development.

Keywords: Zebrafish; Cerebellum; Granule cell; Neuronal migration; Bioimaging; Cell tracing; Fate mapping

Introduction

Many developmental, histological and functional properties of the cerebellum are highly conserved among vertebrates ranging from teleost fish to mammals. This evolutionary conservation includes the position and patterning of the cerebellum under control of the organizer activity of cells at the midbrain–hindbrain boundary (MHB) (Voogd and Glickstein, 1998; Middleton and Strick, 1998; Martinez et al., 1999; Wurst and Bally-Cuif, 2001; Wang and Zoghbi, 2001), its laminar organization comprised by few neuronal cell types of distinct morphology (Nieuwenhuys, 1967; Lannoo et al., 1991; Altman and Bayer, 1997), the characteristic cerebellar feedback circuitry (Wullimann and Northcutt, 1988; Wullimann, 1998; Bengtsson and Hesslow, 2006; Ito, 2006) and, lastly, the role of the cerebellum in coordinating locomotion and contributing to motor learning (Elbert et al., 1983; Roberts et al., 1992; Fiez, 1996; Gao et al., 1996; Boyden et al., 2004; Morton and Bastian, 2004; Rodriguez et al., 2005). Thus, the cerebellum represents one of the highest conserved compartments of the vertebrate brain.

Migration of neuronal precursors is a key step in cerebellar differentiation (Goldowitz and Hamre, 1998; Sotelo, 2004). The precursors of the most common cerebellar interneurons, the granule cells, migrate over long distances. Several fate mapping studies in mouse and chick embryos have revealed their detailed migratory pathways (Miale and Sidman, 1961; Hatten and Heintz, 1995; Lin et al., 2001; Gilthorpe et al., 2002). Granule cells arise from the cerebellar part of a specific germinal zone, called the rhombic lip, which runs along the dorsal aspect of the entire hindbrain ventricle (4th ventricle) (Ben-Arie et al., 1997; Alder et al., 1996). This cerebellar rhombic lip, termed upper rhombic lip (URL), undergoes a reorientation caused by the

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doi:10.1016/j.ydbio.2007.10.024

rotation of the cerebellar primordium during opening of the hindbrain ventricle (Sgaier et al., 2005; Distel et al., 2006). Thus, the URL displays a mediolateral orientation, while the remaining rhombic lip of the hindbrain posterior to the cerebellum, the lower rhombic lip (LRL), displays an anterior–posterior orientation.

Recent fate mapping studies demonstrated that the upper rhombic lip successively produces different neuronal cell types during embryogenesis including neurons of various mesencephalic and hindbrain nuclei, followed by cerebellar deep nuclei and finally granule cells (Machold and Fishell, 2005; Wang et al., 2005; Wingate, 2005; Wilson and Wingate, 2006). Furthermore, lineage tracing of clonally related clusters of URL cells in chick and mouse embryos have shown that the URL gives rise to parasagittal domains of granule cells (Ryder and Cepko, 1994; Mathis and Nicolas, 2003). This suggests that the URL is also spatially patterned along its mediolateral axis. Elegant evidence for such URL subdomains was provided by genetic fate mapping in mouse embryos, where the differential mediolateral extent of expression driven by the promoters from the *engrailed1* and *engrailed2* genes within the cerebellar primordium was exploited (Sgaier et al., 2005). This study showed that granule cell progenitors maintain their mediolateral coordinates after emigration from the URL until the initiation of cerebellar foliation. At this time, granule cell progenitors derived from lateral positions within the URL migrate medially to preferentially populate the posterior-most folia of the mouse cerebellum (Sgaier et al., 2005). Recent genetic experiments suggest that different cerebellar subdomains are generated by a differential sensitivity to *engrailed* gene function (Sgaier et al., 2007). Similar genetic fate mapping studies in mouse for the lower rhombic lip have shown that it is subdivided as well, along its anteroposterior axis (Farago et al., 2006). Such spatiotemporal partitioning of germinal zones provides a powerful means of specifying distinct functional compartments in the mature brain at embryonic stages (Landsberg et al., 2005; Farago et al., 2006).

The zebrafish has become an important model organism to characterize the genetic mechanisms underlying vertebrate brain development. The largely transparent zebrafish embryos are particularly suited for intravital time-lapse studies. In this fashion, molecular mechanisms of neuronal migration (Köster and Fraser, 2001; Haas and Gilmour, 2006; Kirby et al., 2006) and axon pathfinding (Dynes and Ngai, 1998; Hutson and Chien, 2002; Bak and Fraser, 2003) can be studied in the context of brain morphogenesis. Although some genetic studies on the induction of the cerebellar primordium have been performed in the zebrafish (Reifers et al., 1998; Jaszai et al., 2003; Tallafuss and Bally-Cuif, 2003; Foucher et al., 2006), cerebellar morphogenesis, circuitry formation and function remain poorly characterized. In addition, with the exception of Purkinje cells (Lannoo et al., 1991), none of the other cerebellar neuronal cell types, including the cerebellar granule cells, nor patterning within the rhombic lip have been characterized in zebrafish.

In this study we identify the granule cell population of the zebrafish cerebellum. By combining expression analysis with single cell tracing via time-lapse imaging and retrograde labeling, we characterize granule cell differentiation in the context of cerebellar morphogenesis. We also show that the zebrafish cerebellar rhombic lip is spatially patterned similar to mammals. Moreover, we determine that depending on their mediolateral positions within the URL, granule cell progenitors contribute to different functional domains of the mature zebrafish cerebellum. These findings provide an explanation for how zebrafish cerebellar circuitries of different function may be laid down by spatial patterning of germinal zones during embryonic cerebellar development.

Materials and methods

Maintenance of fish

Raising, spawning, and maintaining of zebrafish lines were performed as described previously (Kimmel et al., 1995; Westerfield, 1995). For simplicity, stable transgenic gata1:GFP embryos and adult fish of the strain 781 (Long et al., 1997) will be referred to as gata1:GFP throughout the manuscript.

RT–PCR

Cloning of a partial cDNA of the zebrafish $Gaba_A$-receptor alpha 6 subunit was performed by degenerate RT–PCR using cDNA from adult brains. The following primers were used: Gaba-up: ATGGAATTCACIATIGAYGTITTYT-TYMG and Gaba-low: GACGCATGCRWARCAIACIGCIATRAACCA to amplify an approximately 560-bp fragment, which was cloned into the pCRII-vector (Invitrogen, San Diego, CA). From this vector, a fragment containing a partial cDNA fragment of 423 bp of the zebrafish GabaRα6 subunit (accession number: EF364095) was amplified using the specific primers gabaRa6up: CAAACGTGGATAGATGACCGGCTGAA and gabaRa6lo: ACCTGTGTT-TGACTTCAACCTTTCACTAGAC followed by subcloning into the pSC-B vector (Stratagene, La Jolla, CA).

Morphological stainings

In order to visualize neuropil and the cellular organization of the cerebellum, vital embryo staining and counterstaining of tissue sections were performed by soaking overnight in 0.001% green-fluorescent Bodipy FL C_5-ceramide (D-3521, Invitrogen) or red-fluorescent Bodipy 630/650-X (D-10000, Invitrogen), respectively. For nuclear counterstaining of fixed tissue, DAPI was used at 0.1 μg/ml (Roche, Indianapolis, IN). To remove excess dye, stained specimens were rinsed in PBS/0.1% Tween-20 before images were acquired.

Expression analysis

mRNA in situ hybridization was performed as described (Köster and Fraser, 2006) with the following additions: juvenile and adult brains were dissected after sacrificing zebrafish by an overdose of MS22 (3-aminobenzoic acid ethylester, Sigma, St. Louis, MO) followed by fixation overnight in 4% paraform aldehyde/PBS/0.1% Tween-20. After hybridization, brains were embedded in 4% agarose/PBS. Vibratome sections (Microm HM 650 V, Walldorf, Germany) were cut at 100 μm thickness and mounted on SuperFrostPlus slides (Menzel, Braunschweig, Germany) prior to probe detection.

For immunohistochemical detection the following antibodies were used: polyclonal rabbit anti-GFP (TP401, 1:500, Torrey Pines Biolabs, Houston, TX), mouse anti-GFP (1:500, Molecular Probes, Eugene, OR), polyclonal rabbit anti-phosphohistone H3, PH3, (1:200, Upstate Biotechnology, Lake Placid, NY), mouse anti-human HuC/D (1:500, Molecular Probes), polyclonal rabbit anti-zebrafish Tag-1 (1:1000, received from C. Stürmer), chicken anti-mouse IgG AlexaFluor488-conjugated (1:200, Molecular Probes), goat anti-rabbit IgG Cy2- or Cy5-conjugated (1:200, Jackson ImmunoResearch West Grove, PA) and