ELSEVIER

Brain Research Bulletin 66 (2005) 426–430

BRAIN RESEARCH BULLETIN

www.elsevier.com/locate/brainresbull

Expression domains suggest cell-cycle independent roles of growth-arrest molecules in the adult brain of the medaka, *Oryzias latipes*

Eva Candal *, Vân Nguyen, Jean-Stéphane Joly, Franck Bourrat

JE INRA U1126, INRA/CNRS Group, UPR 2197 DEPSN, CNRS, Institut de Neurosciences A. Fessard, 1 Avenue de la Terrasse, Bâtiment 32-33, F-91198 Gif-sur-Yvette, France

Available online 22 June 2005

Abstract

Teleost fish are unique for their enormous potential to produce new neurons in the adult brain. Nevertheless, the regulation of this adult neurogenesis remains to be characterized. Does it resort to the same molecular mechanisms as those at play in embryonic development? Here, we analyse the expression of the neurogenic gene *Ol-DeltaA* in the brain of medaka (*Oryzias latipes*) embryos and adults. To determine the relationships between neurogenic and growth-arrest genes in the adult brain, we compare the expression domains of *Ol-DeltaA* with those of *Ol-KIP* and *Ol-Gadd45γ*, two well-characterized genes involved in cell-cycle arrest and growth inhibition. While it is widely assumed that genes controlling cell-cycle exit show restricted expression domains next to proliferating cells (in the sites of prospective cell differentiation), we observe highly particular expression domains of *Ol-KIP* and *Ol-Gadd45γ* not associated to proliferating areas of the adult brain, suggesting locally different and cell-cycle independent roles of these molecules in the adult brain.

Keywords: DeltaA; KIP; Gadd45; Cell proliferation; Neurogenesis; Fish

1. Introduction

A major requirement of the vertebrate brain developmental programme is the coordination of cell-cycle regulation, i.e. proliferation versus cell-cycle arrest and cell fate determination [4]. Understanding the molecular mechanisms that link cell-cycle and neurogenesis may be important for understanding human developmental diseases of the brain, many of which are associated with abnormal cell proliferation. The medaka fish *Oryzias latipes* has become a promising model in developmental biology [18]. We have used the optic tectum (OT) of this fish as a model system to identify new regulators of the cell-cycle [15,16] that can then be functionally characterized [2]. The medaka OT is a laminar structure that grows throughout life by the addition of bands of cells from a crescent-shaped proliferative zone (the marginal proliferative zone, mpz) extending from the caudal pole to the ventrolateral and dorsomedial tectum [14]. A whole-mount in situ hybridization (WMISH) screen on the OT of medaka embryos unravelled molecules related to cellular determination (either proneural or neurogenic genes that are expressed in proliferating cells), and also molecules involved in cell-cycle withdrawal, which are expressed in a clearly defined arrest zone at the border between the proliferation and the differentiation zones [15]. Therefore, we isolated *Ol-DeltaA*, a well characterized neurogenic gene that was

Abbreviations: Cb, cerebellum; CbSg, stratum granulare of the Cb; CbSm, stratum molecular of the Cb; CbSp, stratum ganglionare of the Cb; D, dorsal telencephalon; Dlp, area lateroposterior of D; Dm, area medialis of D; fvi, fovea isthmi; GL, glomerular layer of olfactory bulb; Hc, hypothalamus caudal; HD, hypothalamus periventricular dorsal; Hd, dorsal nucleus of the habenula; Hv, ventral nucleus of the habenula; Hy, hypothalamus; ICL, internal granular layer of the olfactory bulb; NDIL, nucleus diffusus of lobus inferioris; NDTL, nucleus difussus of the torus lateralis; NGp, nucleus glomerulosus posterioris; OT, optic tectum; PGZ, periventricular grey zone of the OT; PM, nucleus preopticus magnocellularis; PO, preoptic area; PP, nucleus preopticus parvocellularis; SC, nucleus suprachiasmaticus; Thd, dorsal thalamus; Thv, ventral thalamus; TL, torus longitudinalis; TP, nucleus tuberis posterioris; TS, torus semicircularis; V, ventral telencephalon; Vc, area central of V; Vd, area dorsal of V; Vl, nucleus ventrolateralis of V; VL, area lateralis of the diencephalon; Vs, area supracommissuralis of V; Vv, area ventral of V

* Corresponding author. Tel.: +34 981563100 ext. 13299; fax: +34 981596904.

E-mail address: ecandal@usc.es (E. Candal).

0361-9230/$ – see front matter © 2005 Published by Elsevier Inc.
doi:10.1016/j.brainresbull.2005.05.018

distinctively expressed in the mpz, and two genes expressed in the arrest zone, *Ol-KIP* and *Ol-Gadd45γ*, which code for proteins involved in cell-cycle withdrawal.

An impressive amount of data has been gathered on the role of these molecules during early development, but comparatively little is known on their late expression and roles. It is unclear, in particular, whether adult neurogenesis, which is a general phenomenon in amphibians and fishes where the brain grows continuously, resort to the same molecular mechanisms as those governing the early phases of neurogenesis. Here, we describe the expression patterns of *Ol-DeltaA*, *Ol-KIP* and *Ol-Gadd45γ* in the adult medaka brain. We show that whereas their developmental expression is almost the same, the brain zones where they are expressed in the adult are quite different, suggesting a shift in their roles over time, and pointing out differences between neurogenetic mechanisms in fish embryos and adults.

2. Materials and methods

2.1. In situ hybridization

Sense and antisense digoxigenin-UTP probes for *Histone H2A.Z*, *Ol-DeltaA*, *Ol-KIP* and *Ol-Gadd45γ*, as well as antisense fluorescein-UTP probes for *Histone H2A.Z* were prepared according to Joly et al. [12]. For WMISH, embryos were fixed overnight in 4% paraformaldehyde (PFA) in phosphate-buffered saline 0.12 M (PBS, pH 7.4). They were mechanically dechorionated with fine forceps, dehydrated and stored at −20 °C in methanol. WMISH was performed with an Intavis automat. Proteinase K treatment was adapted to particular developmental stages according to Joly et al. [12], but for adult brains this treatment was extended up to 45 min and detection up to 24 h. Control sense probes did not lead to any detectable signal. Whole embryos were mounted in 1% methylcellulose in tap water (Sigma, stored at 4 °C) and observed under a SV11 Zeiss dissecting microscope. Some hybridized and post-fixed embryos and adult brains were embedded in wax and serially sectioned at 8 μm on a rotary microtome. Wax sections were counterstained with nuclear fast red. Pictures were taken with a Nikon DXM 1200 digital camera mounted on a Leica DRM microscope. Double WMISH was performed as described by Hauptmann and Gerster [11].

2.2. Histology and nuclear staining

Dechorionated embryos, juveniles and adults were anaesthetized and immediately fixed by immersion in Clark's solution (3:1 ethanol 100%: acetic acid) for 1 h at room temperature (RT), dehydrated, wax-embedded and serially sectioned (8 μm) on a rotary microtome. Sections were dewaxed in xylene and re-hydrated through a graded series of alcohols. Endogenous peroxidase was blocked with 3% H_2O_2 in PBS pH 7.4 for 30 min at RT. Samples were then processed using an anti-proliferating cell nuclear antigen (anti-PCNA, Eurodiagnostica, 1:500) followed by biotinylated secondary antibody and the Avidin Biotinylated horseradish peroxidase complex (VECTASTAIN Elite ABC kit, Vector Laboratories). DAB was used as peroxidase substrate. Photographs were made as mentioned above.

2.3. Double-labeling Ol-KIP mRNA proliferating cell nuclear antigen

In situ hybridization was performed as described above. After the developing step, embryos and adult brains were fixed in Clark's solution (10 and 20 min, respectively), dehydrated, wax-embedded and processed for PCNA immunohistochemistry. To optimize antigen retrieval, microwave pre-treatment was performed as described by Nguyên et al. [15].

3. Results

In our WMISH screen we isolated a clone that exhibited a horseshoe-like expression in the mpz of the tectum (Fig. 1A and B). Sequence analysis revealed that it codes for *Ol-DeltaA*, a member of the family defined by *Drosophila Delta*, a neurogenic gene that prevents proliferating neural cells from differentiating [8]. Two other genes were selected on the basis of their expression in the arrest zone located at the border of the mpz. Firstly, *Ol-KIP* (Fig. 1C and D), *O. latipes*-kinase inhibitor protein [16], ortholog of the cyclin-dependent kinase inhibitor $p57^{KIP2}$ the role of which in cell-cycle withdrawal coupled to cell determination–differentiation has been largely reported [3,6]. Secondly, *Ol-Gadd45γ* (Fig. 1E and F), ortholog of mammalian Gadd45γ, a member of the Gadd45 family of growth-arrest and DNA-damage inducible genes [2], that has been shown to play an important role in coordinating cell fate decisions (cell-cycle arrest or apoptosis) during neurogenesis. The proliferative zone was identified either by immunohistochemistry with an antibody against the proliferating cell nuclear antigen (Fig. 1D), or by WMISH with an antisense *Histone H2A.Z* probe (Fig. 1F), which is highly expressed in proliferating cells (in humans, the amount of the *Histone h2A.Z* transcript is greatly decreased as proliferating cells become quiescent, [10]).

The expression domains of *Ol-DeltaA* were first analysed on embryos at different developmental stages (Fig. 1H) and compared with brain sections at the same developmental stage immunostained for PCNA (Fig. 1G). During brain development, the expression of the neurogenic gene *Ol-DeltaA* is detected in all proliferative zones and decreases as neural differentiation processes (Figs. 1I and 2A–G). In the adult brain, *Ol-Delta A* was expressed periventricularly in the areas in which adult cell proliferation in the medaka brain is well documented [16]: in the dorsal and ventral telencephalon (Fig. 2A and B), in the habenula (Fig. 2C), in the