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PROLIFERATION ZONES IN THE BRAIN OF ADULT FISH *AUSTROLEBIAS* (*CYPRINODONTIFORM: RIVULIDAE*): A COMPARATIVE STUDY

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Abstract—In contrast with mammals, adult fish brains exhibit an enormous potential to produce new cells. Proliferation zones, however, have been described in only a few species, hindering comparisons among genuses and orders. Here we analyzed brain cell proliferation in annual teleostean fishes *Austrolebias* (*Cyprinodontiform:* Rivulidae). Immunocytochemistry against 5-bromo-2′-deoxyuridine (BrdU) was quantitated and mapped 24 h after injection in three species with different phylogenetic positions or habitats. All species had similar brain anatomy and total volume, but olfactory bulbs, *torus longitudinalis* and *cerebellum* were of different sizes in different species. Cell proliferation was found throughout the brain. Three-D reconstructions provided evidence for contiguity along the rostro-caudal axis and concentration in the vicinity of the ventricles. Brain regions analyzed exhibited high mitotic activity, and the *torus longitudinalis* had the highest volume-normalized proliferation index. *A. affinis* exhibited the highest normalized proliferation indexes in visual regions but the lowest in olfactory bulb. *A. reicherti* showed an inverse pattern, suggesting that these species have a different hierarchy of sensorial modalities that could be related to phylogeny or habitat. Double immunostaining against BrdU and cell-type specific markers was performed to determine the fate of proliferating cells. A widespread gliogenesis was evidenced. Few cells positive for both BrdU and the neuronal marker HuC/D were found in the brain of the three species, demonstrating neurogenesis in the adult *Austrolebias* brain. Summarizing, adult members of the three species showed similar brain anatomy and cell proliferation patterns. Among species, volume-normalized proliferation indexes varied in regions involved in different sensory modalities. To our knowledge, this is the first report showing proliferating cells with neuronal markers as earlier as 24 h after BrdU injection.

Key words: Austrolebias brain, proliferation zones, neurogenesis, quantitative analysis.

In all vertebrate species examined, the production of new neurons in the central nervous system persists in adulthood (Zupanc, 2001a). However, an evolutionary trend towards a reduction in both the number of proliferation zones and newborn cells has been revealed in more evolutionary groups. In mammals, adult neurogenesis and proliferation zones are restricted to very few regions, such as the forebrain subventricular zone, the olfactory bulb and the hippocampal complex (for reviews, see Doetsch and Scharff, 2001; Gage, 2002; Rakic, 2002; Lledó et al., 2006). Conversely, in amphibians (Polenov and Chetverukhin, 1993), reptiles (Pérez-Cañellas et al., 1997; Font et al., 2001; Fernández et al., 2002), birds (Goldman and Nottebohm, 1983; Álvarez-Buylla et al., 1990; Arai and Saito, 1995; Ling et al., 1997), and fishes (Birse et al., 1980; Anderson and Waxman, 1985; Zupanc and Horschke, 1995; Ekström et al., 2001), many more proliferative zones have been described. In addition, postnatal neurogenesis and gliogenesis are more pronounced and persist for longer periods during life. Such differences between mammals and other vertebrates provide an opportunity to examine adult brain cell proliferation and neurogenesis from a comparative point of view.

Studies with freshwater fishes showed many new cells and neurons in several brain regions. Moreover, brain cell proliferation rates are one or two orders of magnitude higher in teleosts than in mammals (Zupanc and Zupanc, 2006). New born cells likely accumulate in existing populations thereby contributing to the continuous growth of fish brain (Raymond and Easter, 1983; Zupanc et al., 1996). New neurons also replace ones lost, enabling regeneration, a remarkable difference between fishes and mammals which commonly are unable to regenerate the nervous system (Zupanc, 1999).

Austrolebias (euteleosts, Cyprinodontiform: Rivulidae) are an endemic and numerous group of annual Neotropical fishes that inhabit temporary ponds that dry out seasonally (Vaz-Ferreira and Melgarejo, 1984; Nelson, 2006). Adaptations for maintaining *Austrolebias* species include high metabolic rates and an elaborate courtship which ends in the deposition of drought-resistant eggs, capable of going

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Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; CB, cerebellum; Cc, corpus cerebelli; EG, eminentia granularis; OB, olfactory bulb; Tel, telencephalon; TL, torus longitudinalis; TO, tectum opticum; Vc, valvula cerebelli; VT, telencephalic ventricle.

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through diapauses in the substrate when the adult population has died (García et al., 2004). The extensive karyotypic divergence of *Austrolebias* is of interest from an evolutionary standpoint, thus, this genus became an appropriate model to study speciation (García et al., 1995, 2002, 2004). The short lifespan and diversity of habitats together with phylogenetic divergence, indicated that *Austrolebias* could be a novel and powerful model to study postnatal brain cell proliferation and neurogenesis from a comparative point of view, and perhaps to correlate it with evolutionary events (Zupanc, 2001b). The comparative analysis of proliferation zones in *Austrolebias* could shed light on the plasticity of the central nervous system proliferative/neurogenic capacity, and also to give cues to understand brain cell proliferation within this genus. Thus, we investigated if differences among species or habitats might be reflected in either anatomy or cell proliferative capacity of the *Austrolebias* brain.

In the present study, brain gross anatomy and proliferative zones of three species of adult *Austrolebias* were studied. Mapping, 3-D reconstruction and quantitative analysis of the main proliferative zones was made in olfactory bulb (OB), telencephalic ventricles (VT), *torus longitudinalis* (TL), *tectum opticum* (TO) and *cerebellum* (CB). An estimation of volume-normalized proliferation index was made in OB, TL, TO and CB. A study of double BrdU and cell-type specific marker immunostaining was done to show fate of proliferating cells.

EXPERIMENTAL PROCEDURES

Animals

Three species of *Austrolebias* were used in this study. Specimens were obtained from unrelated Uruguayan sites. *A. affinis* were collected in Durazno, whereas *A. charrua* and *A. reicherti* were obtained in Treinta y Tres (300 km from Durazno). Fishes were collected with hand nets, in temporary ponds close to major rivers. Two or 4 days prior to experiments, animals were transferred to a freshwater aquarium maintained at 19 °C under natural photoperiod and fed daily with live *Tubifex sp*. Animals used were adult males between 6 and 8 months old (Arezo et al., 2005). All experiments were conducted under the guidance of the local Committee for Animal Care and Research (CHEA, UdelaR), which follows NIH guidelines for maintenance and use of laboratory animals.

Anatomical comparison

To improve the resolution and comparison of anatomical features, serial sections of individual whole brains of the three species were made. Each section was photographed by using a Nikon light microscope equipped with a digital camera, Coolpix 995 (Nikon, Japan). Images were analyzed individually and served as topographic references for comparison between species. Afterward 1024×1024 images of serial section of two brains of each species were analyzed by BioVis3D software (http://www.biovis3D.com). Each serial section profile was drawn and then stacked to obtain a topographic representation of the brain. Employing the BioVis3D measure tool, volumes of total brain and manually drawn structures such as olfactory bulbs, *tectum opticum*, *torus longitudinalis* and *cerebellum* were calculated for the three species.

5-bromo-2′-deoxyuridine (BrdU) treatments and immunocytochemistry

Six adult fishes from each species (n=18) were injected i.p. with a single dose (100 mg/kg body weight) of BrdU (Sigma-Aldrich, Milwaukee, WI, USA) dissolved in 0.6% sodium chloride. Twenty four hours post-injection, fishes were deeply anesthetized by adding a 1:1000 v/v solution of 10% Eugenol (Sigma) to the aquarium water. Animals remained in the aquarium until opercular movement ceased. At that time, each fish was intracardially perfused with saline solution to wash the vascular system and then fixed with paraformaldehyde (10% in 0.1 M phosphate-buffer (PB), pH 7.2–7.4). Brains were removed and kept in the same fixative solution for another 2 h at 4 °C. Then, tissue was transferred to PB overnight. Fixed brains were serially sectioned (60 μm thickness) in a Vibratome S1000 (Leica) and sections were transferred to multi-well plates for further free-floating immunocytochemistry processing. To visualize the BrdU incorporated to cells in S-phase, DNA-denaturation was performed by incubating sections in 2 N HCl-0.3% Triton X-100 (dissolved in PB) for 45 min at room temperature. After several washes with PB, floating sections were incubated overnight at 4 °C with a 1:500 dilution of a monoclonal mouse anti-BrdU antibody (Dako, Copenhagen, Denmark). After through buffer rinses, the sections were incubated in 1:300 anti-mouse IgG conjugated with horseradish peroxidase (Chemicon, USA & Canada), after an hour activity was visualized with addition of 3,3′-diaminobenzidine (Sigma). To determine optimal conditions for immunostaining, intestine sections from rats injected with BrdU or PB were used as positive and negative controls, respectively. All sections were mounted with glycerol mounting medium and photographed.

When double-immunolabeling was done, after DNA-denaturation, some floating sections were incubated with the primary anti-BrdU antibody together with anti-HuC/D (1:200, Invitrogen) or anti-glial fibrillary acidic protein (GFAP, 1:500, Sigma) or anti-S-100 (1:500, Sigma) antibody, respectively. Recognition of respective labeling was done with secondary antibodies conjugated with fluorescent markers Alexa 488 and Alexa 633 (Invitrogen, USA), both diluted 1:2000 in 0.3% Triton X-100-PB. Double immunostaining was done with five animals of each species and all regions were analyzed.

Fluorescent images were obtained using a confocal FV300 Olympus microscope, and sequential imaging and multi-plane view analysis were performed to determine co-localization of signals.

3-D visualization of proliferative zones

Two brains of each species were used for 3-D mapping of proliferative zones. Brains were serially sectioned in the transverse plane, and BrdU immunostaining was performed and developed with diaminobenzidine. Optical images were analyzed by BioVis3D software. The profile of each section was drawn and the regions bearing BrdU-labeled nuclei were represented by dots of different colors, each one identifying a proliferative zone and representing two to five BrdU+ nuclei. Then, section profiles were stacked to obtain the topographic representation of the proliferative zones along the brain.

Quantitative analysis of proliferation and estimation of volume-normalized proliferation indexes

Total positive BrdU nuclei of consecutive sections corresponding to the levels containing the olfactory bulb, telencephalic ventricle, *torus longitudinalis*, *tectum opticum* and *cerebellum* were counted in all animals processed (six per species, 18 overall). The total averaged number of BrdU+ cells in each region per animal was plotted using Origin 8.0. Descriptive statistical analysis was made with Sigma Stat2.0.