European Journal of Neuroscience, Vol. 25, pp. 1030–1040, 2007 doi:10.1111/j.1460-9568.2007.05366.x

Sex differences in adult cell proliferation within the zebrafish (*Danio rerio*) cerebellum

Konstantinos Ampatzis[1] and Catherine R. Dermon[1,2]
[1]Department of Biology, University of Crete, Heraklio, 71409, Crete, Greece
[2]Department of Biology, University of Patras, 26500, Rion, Greece

Keywords: apoptosis, chain migration-like, neurogenesis, neuronal plasticity, parvalbumin

Abstract

It has been reported that neurons generated in the adult brain show sex-specific differences in several brain regions of lower vertebrates and mammals. The present study questioned whether cell proliferation and survival in the adult zebrafish (*Danio rerio*) cerebellum, the most mitotically active area of adult teleost brain, is sexually differentiated. Adult zebrafish were treated with the thymidine analogue 5′-bromo-2′-deoxyuridine (BrdU) and allowed to survive for 24 h (short-term) and for 21 days (long-term). BrdU immunohistochemistry allowed visualization of cells incorporating BrdU at the S phase of mitosis. At short-term survival, male zebrafish had a higher number of labelled cells at proliferation sites of the molecular layer of corpus cerebelli (CCe) and the granular layer of the caudal lobe of the cerebellum (LCa) than did females. In long-term survival, BrdU-positive cells were found at their final destination, but only the granular layer of the medial division of the valvula cerebelli showed sex-specific differences in the number of labelled cells. This higher mitotic activity in male cerebellum might be related to sex-specific motor behaviour observed in male zebrafish. To investigate the role of programmed cell death, the terminal deoxynucleotidyl-mediated dUTP nick-end-labelling (TUNEL) method was applied. The vast majority of apoptotic figures occurred in the granular cell layer of valvula and CCe, only in a few cases within the BrdU-retaining cells. Apoptosis was found specifically at the sites of the final destination of proliferating cells, indicating that the close relation of cell birth and death might represent a possible plasticity mechanism in the adult zebrafish cerebellum.

Introduction

Adult neurogenesis is known to show sex differences in brain regions in birds and mammals, correlating differences in sex-specific behaviour with anatomical sexual dimorphisms. Adult songbirds have proved a significant model of sexual dimorphism underlying male sonic courtship behaviour and song production (Goldman & Nottebohm, 1983; Nordeen & Nordeen, 1989; Rasika *et al*., 1994; Hidalgo *et al*., 1995). It is also suggested that, in mammalian brain, adult neurogenesis is sexually differentiated in the dentate gyrus where newly generated neurons differentiate into hippocampal granule cells, with the female animals exhibiting higher numbers of newborn cells (Gould *et al*., 1999; Tanapat *et al*., 1999). Recently, it has been suggested that the number of newly generated cells reaching the adult anterior accessory olfactory bulb is also sexually differentiated, with male rats exhibiting higher numbers of newborn cells (Peretto *et al*., 2001). In addition to neurogenesis and cell survival, it has been suggested that sex differences in cell numbers in several neural structures develop through sex-steroid-sensitive mechanisms that regulate apoptosis (Nordeen *et al*., 1987; Kirn & DeVoogd, 1989; Kirn & Schwabl, 1997; Stokes *et al*., 2004; Tsukahara *et al*., 2004).

In comparison to birds and mammals, cell proliferation in the central nervous system of adult fish is found at considerably higher levels (Alvarez-Buylla, 1990; Dawley *et al*., 2000; Zikopoulos *et al*., 2000; Byrd & Brunjes, 2001; Ekström *et al*., 2001; Zupanc, 2001) throughout life (Ekström *et al*., 2001). While there is evidence for sex differences in cell proliferation in fish hypothalamus (Zikopoulos *et al*., 2000, 2001), an area related to reproductive behaviour, as in mammals (Galea & McEwen, 1999), there is no knowledge on possible sex differences in brain structures not directly related to reproduction, such as the teleost cerebellum. In addition to motor coordination (Paul & Roberts, 1979; Roberts *et al*., 1992), teleost fish cerebellum is involved in spatial cognition and emotional learning (Yoshida *et al*., 2004; Rodriguez *et al*., 2005) and in memory processes (Lalonde & Botez, 1990) and, like the cerebellum of mammals (Supple & Kapp, 1993; Gherladucci & Sebastiani, 1996), plays an essential role in the classical conditioning of emotional responses (Rodriguez *et al*., 2005).

The hypothesis that sex-specific differences may underlie structural plasticity during adulthood by influencing cell proliferation and survival was tested in zebrafish teleost cerebellum, an area exhibiting high proliferative activity where the vast majority of the new cells develop into granule cell neurons in most adult teleosts (Zupanc *et al*., 1996; Zupanc *et al*., 2005). The cytoarchitecture of zebrafish cerebellum resembles that observed in other vertebrates (Meek & Nieuwenhuys, 1998), except for the presence of eurydendroid cells, located in the Purkinje cell layer, which are the cerebellar output neurons. The present study aimed to determine possible sex differences in mitotic as well as in apoptotic activity in the main compartments of the adult zebrafish cerebellum, by means of 5′-bromo-2′-deoxyuridine (BrdU) and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labelling (TUNEL) methods.

Correspondence: Professor C. R. Dermon, [2]Department of Biology, as above.
E-mail: dermon@biology.uoc.gr, dermon@upatras.gr

Received 23 June 2006, revised 6 December 2006, accepted 18 December 2006

Materials and methods

Animals and BrdU administration

Adult zebrafish *Danio rerio* (cyprinidae, Teleostei; $n = 24$), obtained from our aquaculture laboratory (Department of Biology, University of Crete), weighing 0.8 ± 0.195 g (mean ± SEM), were randomly chosen and used for analysis. The sex of the fish was initially determined based on the observation of the sex-specific somatic characteristics. Male fish were kept separately from female individuals in the laboratory on a 12 : 12 h light : dark cycle at a controlled water temperature of 28.5 ± 0.2 °C (mean ± SEM) as described in The Zebrafish Book (Westerfield, 1995), in 15-L plastic aquaria, for 4 days. The fish were anaesthetized with 0.02% tricaine methanesulphonate (MS 222; Sigma, Deisenhofen, Germany) and injected intraperitoneally with a single dose of a saline–BrdU solution (Sigma; 0.2 mg/g body weight). BrdU is a nonradioactive analogue of thymidine, incorporated into the DNA of proliferating cells during the S phase of mitosis (Miller & Nowakowski, 1988). Fish were then allowed to survive for 24 h (short-term survival) or 21 days (long-term survival). All experimental procedures were in accordance with the European Communities Council directive (86/609/EEC) for the care and use of laboratory animals and approved by the University of Crete research committee.

Tissue sampling

Following 24 h BrdU ($n = 10$) or 21 days of BrdU ($n = 14$) injection the fish were anaesthetized with lethal dose of 0.04% MS 222 and intracardially perfused with saline (0.9% NaCl) followed by 4% paraformaldehyde (Sigma) freshly depolymerized in phosphate-buffered saline (PBS; 0.01 M, pH 7.4). The sex of the animals was identified by their gonad morphology using methylene blue histological preparations. The brains were carefully removed, postfixed for 2 h at 4 °C, cryoprotected overnight with 20% sucrose in fixative solution at 4 °C, embedded with tissue freezing medium (Jung, Leica Instruments, Wetzlar, Germany) and rapidly frozen in dry ice-cooled isopentane (2-methyl butane; Sigma) at ∼−35 °C and were stored at −80 °C until use. The brains were cut in a cryostat (Leica, CM1500) and serial coronal 20 μm sections, spaced 80 μm apart, were mounted on gelatin-coated slides, air-dried for 1 h and processed for single-labelling and double-labelling immunohistochemistry. BrdU and TUNEL single-labelling experiments were performed in adjacent sections. Double-labelling experiments were performed in the long-term animals.

BrdU and TUNEL immunohistochemistry

The avidin–biotin–HRP complex (ABC) method was used to visualize specific staining with diaminobenzidine as chromogen, using a monoclonal antibody against BrdU (Becton Dickinson, San Jose, CA, USA).

To visualize incorporated BrdU, DNA denaturation was performed by incubating the sections in 2 N HCl for 30 min at 37 °C, followed by thorough washing in PBS and then H_2O_2 dissolved in PBS for 10 min at room temperature (RT) to inhibit endogenous peroxidase activity. The sections were then washed three times for 5 min in PBS. Nonspecific protein binding sites were blocked with 0.15% normal horse serum with 5% bovine serum albumin and 0.5% Triton X-100 in PBS for 30 min at RT. Sections were incubated with mouse anti-BrdU (dilution 1 : 100) in 0.5% Triton X-100 in PBS at 10 °C for 18–20 h in a moist chamber. After thorough buffer rinses the sections were incubated in biotinylated horse antimouse IgG secondary antibody (Vector Laboratories, Burlingame, CA, USA; dilution 1 : 200) in PBS for 2–3 h at RT. The sections were then washed three times in PBS with 0.5% Triton X-100 and incubated with Vectastain Elite ABC reagent (Vector Laboratories; dilution 1 : 100 A and 1 : 100 B) in PBS with 0.5% Triton X-100 for 1 h in the dark at RT and washed with PBS.

In situ TUNEL was used to reveal the spatial distribution of apoptotic cells in adult zebrafish brain. This method distinguishes apoptosis from necrosis by specifically detecting DNA cleavage and chromatin condensation associated with apoptosis. Brain sections were processed for detection of DNA fragments using the ApopTag Peroxidase *in situ* detection kit (Chemicon International Inc., Temecula, CA, USA) to reveal the apoptotic activity. The sections were post-fixed in precooled ethanol : acetic acid, 2 : 1, solution for 5 min at −20 °C, washed twice with PBS and incubated with 3% H_2O_2 solution for 5 min at RT in order to quench endogenous peroxidase. Sections were then rinsed twice with PBS. After application of the kit's equilibration buffer, working-strength TdT enzyme was applied to the sections for 1 h at 37 °C. TdT catalyses a template-independent addition of nucleotide triphosphates to the 3′-OH ends of DNA. The incorporated nucleotides form an oligomer composed of digoxigenin-conjugated nucleotide in a random sequence. The sections were then washed with PBS, and antidigoxigenin peroxidase conjugate antibody was applied to slides for 30 min at RT.

The BrdU and TUNEL labelling were revealed with 3,3′-diaminobenzidine (DAB; Vector Laboratories). Methyl Green and Cresyl Violet were used as counterstains (Sigma, 1% Methyl Green in double-distilled (dd)H_2O, or 0.5% Cresyl Violet in ddH_2O) in order to facilitate and enhance the identification of brain regions. The sections were then dehydrated, cleared with xylene and coverslipped with Entellan (Merck, Darmstadt, Germany).

To detect nonspecific labelling, adjacent sections were incubated with PBS in the absence of the primary or secondary antibody. No labelled cells were observed under these control conditions.

BrdU- and TUNEL-positive cells were easily recognised and identified by dense homogeneous staining of their nuclei and could be clearly differentiated from the possible expression of endogenous peroxidase activity by erythrocytes.

Double-labelling immunohistofluoresence

Double-labelling immunohistofluorescence was used to characterize the newborn cells in the long-term experiments. For parvalbumin (PV) expression in the adult zebrafish cerebellum we used a completely characterized (Celio *et al*., 1988) monoclonal antibody (IgG1), which specifically stains the ^{45}Ca-binding spot of PV and has been used previously in mammalian (Celio, 1990; Bu *et al*., 2003), avian (Wild *et al*., 2005) and teleostean (Alonso *et al*., 1992; Porteros *et al*., 1998; Crespo *et al*., 1999) brain. In addition, we used an intermediate filament protein, glial fibrillary acidic protein (GFAP), specific for astrocytes and radial glia as well as some types of ependyma cells in most vertebrates (Levitt & Rakic, 1980; Bodega *et al*., 1990, 1993, 1994; Eng *et al*., 2000).

Sections were incubated with a cocktail of primary antibodies for BrdU (rat anti-BrdU, dilution 1 : 100; Accurate Chemical and Scientific Corp., Westbury, NY, USA) and PV (mouse anti-PV, dilution 1 : 2500; Swant) or GFAP (mouse anti-GFAP, dilution 1 : 100; Sigma) in 0.5% Triton X-100 in PBS at 10 °C for 18–20 h. Secondary antibodies, antirat Alexa Fluor 488 and antimouse Alexa Fluor 568 (diluted 1 : 500 in 0.5% Triton X-100 in PBS; Molecular

Probes, Leiden, the Netherlands), were used simultaneously for 2 h at RT in the absolute dark. Adjacent sections were used for single labelling of BrdU or PV and detection of nonspecific labelling in the absence of the first or second antibody.

In order to examine the neuronal differentiation of the proliferating cells, additional BrdU–HuC/D (see below) and BrdU–neuronal nuclei (NeuN) double-labelling experiments were performed. HuC/D regulatory RNA binding proteins function early in neuronal differentiation (Marusich *et al*., 1994) while the NeuN antibody is a specific marker for postmitotic neurons (Mullen *et al*., 1992). These neuronal markers HuC/D (Byrd & Brunjes, 2001; Mueller & Wullimann, 2002; Grandel *et al*., 2006) and NeuN (King *et al*., 2004) have been used in many studies in the nervous system of fish species, including the zebrafish. Briefly, following DNA denaturation (2 N HCl for 30 min at 37 °C), the sections were incubated either with HuC/D (mAb 16A11, diluted 1 : 100; Molecular Probes) or NeuN primary antibodies (diluted 1 : 200; Chemicon) in PBS with 0.5% Triton X-100 overnight at 10 °C. An antimouse Alexa Fluor 568 (Molecular probes; diluted 1 : 500 in 0.5% Triton X-100 in PBS) was added to sections for 3 h at RT in the dark. The sections were then incubated with the monoclonal rat anti-BrdU (1 : 200; Accurate Chemical) for 14 h at 10 °C followed with a antirat Alexa Fluor 488 (diluted 1 : 500 in 0.5% Triton X-100 in PBS; Molecular probes) for 3 h at RT. The sections were thoroughly rinsed in PBS and coverslipped with fluorescent hard medium (H-1400; Vector).

To detect whether proliferating cells contain fragmented DNA the TUNEL reaction was performed, as previously described except for the use of antidigoxigenin antibody which is conjugated to a fluorescein reporter molecule (ApopTag Kit, S7110; Chemicon), to obtain fluorescent TUNEL nuclei (green). This kit was used following the manufacturer's guidelines. After that the sections were processed for BrdU immunohistochemistry using a monoclonal mouse anti-BrdU (1 : 100; Becton Dickinson) and an antimouse Alexa Fluor 568 (1 : 500; Molecular probes) antibodies. The sections were rinsed extensively with PBS and coverslipped with the fluorescent hard medium (H-1400; Vector).

Quantification of labelled cells

Single-labelling BrdU and TUNEL experiments of short- and long-term survival were used for quantification of cell proliferation, survival and apoptosis. Cell counts of BrdU and TUNEL labelling were obtained within each cerebellum ($n = 5$ for each sex and survival group) in all sections sampled (26 equidistantly 80 μm-spaced serial sections along the entire rostrocaudal axis of the cerebellum) using a microscope (Eclipse E800; Nikon, Tokyo, Japan) at 600× magnification with the aid of a camera lucida. Labelled cells were identified by the intense nuclear labelling exhibiting the characteristic DAB reaction. The BrdU- and TUNEL-positive nuclei were counted as they came into focus while scanning through 20 μm of the tissue section thickness (*z*-axis). The total area of the cerebellum region analysed was determined via a colour 3CCD Sony DXC-950P camera to a PC (Microsoft Windows XP) with the aid of an image analysis system (Scion Image B4.0.2; Scion Corp., Frederick, MD, USA). The numbers of BrdU- or TUNEL-positive cells per cerebellar region were expressed as the fraction of BrdU- or TUNEL-positive cells in each region per unit area of this region (BrdU- or TUNEL-positive cell profiles). Labelled cells were localized according to the atlas of Wullimann *et al*. (1996). Differences in numbers of positive cells between sexes (male, female) and survival time (short-term, long-term) were statistically compared using two-way ANCOVA (using the body weight as a covariate) with Statgraphic Plus v5.0 (Statistical Graphics Corp.).

In addition, in order to test whether sex differences characterize the total number of granule cells, we quantified counterstained cells using a specific counting frame (0.04 × 0.04 mm, 0.0016 mm^2 area) with exclusion of the edges, in 10 representative serial coronal sections throughout the entire rostral–caudal cerebellar axis, in six male and six female individuals. Differences in total number of granule cells in granular cell layers of the cerebellum between sexes were statistically compared using *t*-tests (two-tailed hypothesis; Statgraphic Plus v5.0, $P < 0.05$).

Size and luminosity of the figures were modified using the Macintosh version of Adobe Photoshop CS2 (Adobe Systems Inc., San Jose, CA, USA). The graphs were prepared using Corel Draw 11 (Corel, Dallas, TX, USA) for Macintosh. Volume (3-D) reconstructions of BrdU-positive cells were performed with the VolumeJ (Abramoff & Viergever, 2002) extension for the Macintosh version of ImageJ (Rasband, 2004).

Results

The zebrafish cerebellum, unlike that of other vertebrates, has three parts: the valvula cerebelli (Va), which is divided into medial (Vam) and lateral (Val) subdivisions, the corpus cerebelli (CCe), and the vestibulolateralis lobe, which is formed by the medial caudal lobe (LCa) and the paired lateral eminentia granularis (EG). The basic features of the teleost cerebellum are the same as those of amniote vertebrates, not only in CCe but also in Val and Vam (Kotchabhakdi, 1976; Meek & Nieuwenhuys, 1998). In the Va, the Purkinje zone is bent and some of the granular cells are located on the surface of the Va; the Purkinje-free boundary between the granular cell layer (-gr) and the molecular cell layer (-mol) is very complex (Wullimann *et al*., 1996; Miyamura & Nakayasu, 2001).

Significant populations of BrdU-positive cells were found in the adult zebrafish cerebellum after 24 h of BrdU administration. The vast majority of them were located close to the midline of the medial CCe-mol and in most cases formed a chain moving away towards the lateral surface to the corresponding granular cell layer (Fig. 1). In the midline, the granular cell layer of CCe forms a tip, invading the molecular layer. In this specific area between the molecular and granular layer a small number of round-shaped BrdU-labelled cells were observed to remain in their position. One day (24 h) following

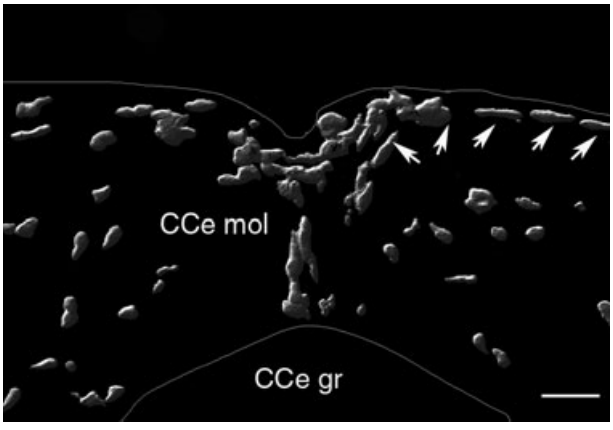

FIG. 1. 3-D volume reconstruction of a significant proliferation site in CCe-mol, using the software VolumeJ. The arrows indicate the chain migration-like pattern of newly born cells. Scale bar, 0.1 mm.

BrdU administration, only 5% of the labelled cells had completed their migration into the granular cell layer. In addition, ~70% of the BrdU-positive cells were elongated in shape and appeared to be migrating, while 25% of the newly born cells remained in the proliferation area. In addition, few positive cells were found over the dorsal part of the CCe-gr (Fig. 2I).

In the Val and Vam the majority of the mitotic activity occurred in the molecular layer (Vam-mol, Val-mol) as in the CCe (Fig. 2, IA and B), and only a few cells were located in the granular layer (Vam-gr, Val-gr). Unlike other cerebellar regions, the LCa-gr included higher mitotic activity (Figs 2, IC, 4C and D, and 5A) than the LCa-mol. It is interesting to note that the crista cerebellaris (CC; Fig. 2, IC), an area with close interrelations with the cerebellum, included low levels of mitotic activity.

In the Va and the CCe of adult zebrafish brain, almost all labelled cells had migrated away from the proliferating areas of the respective molecular layers, into the associated granular layers, at a survival time of 21 days (Figs 2II and 3A–E). In LCa, no obvious migrating pattern could be obtained and labelled cells remained in the granular layer, where they were produced.

Double-labelling experiments performed 21 days post-BrdU administration (in the long-term survival groups) revealed that a significant population of BrdU-positive cells in the granule cell layer were also HuC/D- and NeuN-positive (Fig. 3D–E), in agreement with previous studies supporting the idea that the majority of the proliferating cells in adult zebrafish cerebellum become granule cells (Zupanc *et al*., 2005). It is important to note that, while the neuronal markers used showed similar staining of cerebellar granule cells, the majority of Purkinje and eurydendroid cells in the ganglionic layer were labelled only by HuC/D, not by NeuN, in all cerebellar compartments (Fig. 3D and E). Not all BrdU-positive cells expressed the neuronal markers tested and, while there is no evidence as to whether a subpopulation of these adult-generated cells are glial, additional double-label immunohistochemistry was performed to further determine the type of adult-generated cells. Detailed examination of double-label experiments with BrdU–PV and BrdU–GFAP revealed that none of the BrdU-positive cells were labelled with the neuronal marker PV (Fig. 3A–C) or with the glial marker GFAP (Fig. 3F). In all divisions of the cerebellum, PV-immunostained cells and fibres were observed in all three layers of the cerebellum. Strong PV immunoreactivity was observed in Purkinje cell somata and in the dendritic trunks in the molecular layer (Fig. 3A–C). In addition, some PV-immunostained axons of the cells of the ganglionic cell layer were also found in the granular cell layer (Fig. 3A–C). The eurydendroid cells located in the ganglionic cell layer were observed to be surrounded by PV-positive axon terminals.

In both male and female adult zebrafish cerebellum, a few radial glial fibres were observed but no GFAP-positive cell bodies were found throughout the layers of the cerebellum. The majority of the GFAP-positive fibres were found in the molecular layer of the cerebellum (Fig. 3F). In agreement, a monoclonal antibody C-4 (which stains radial glia) labels long processes in the molecular layer in adult zebrafish cerebellum (Tomizawa *et al*., 2000). A small number of individual GFAP-immunostained structures were also observed in the granular cell layer. Detailed observation of the sections revealed that the Va included most of the immunoreactive GFAP fibres. In few cases, GFAP-positive fibres were found in close apposition to BrdU-positive cells, suggesting that radial glial fibres were used by migrating young cells in the adult zebrafish cerebellum, guiding migrating young cells as during development (Levitt & Rakic, 1980). In most cases, though,

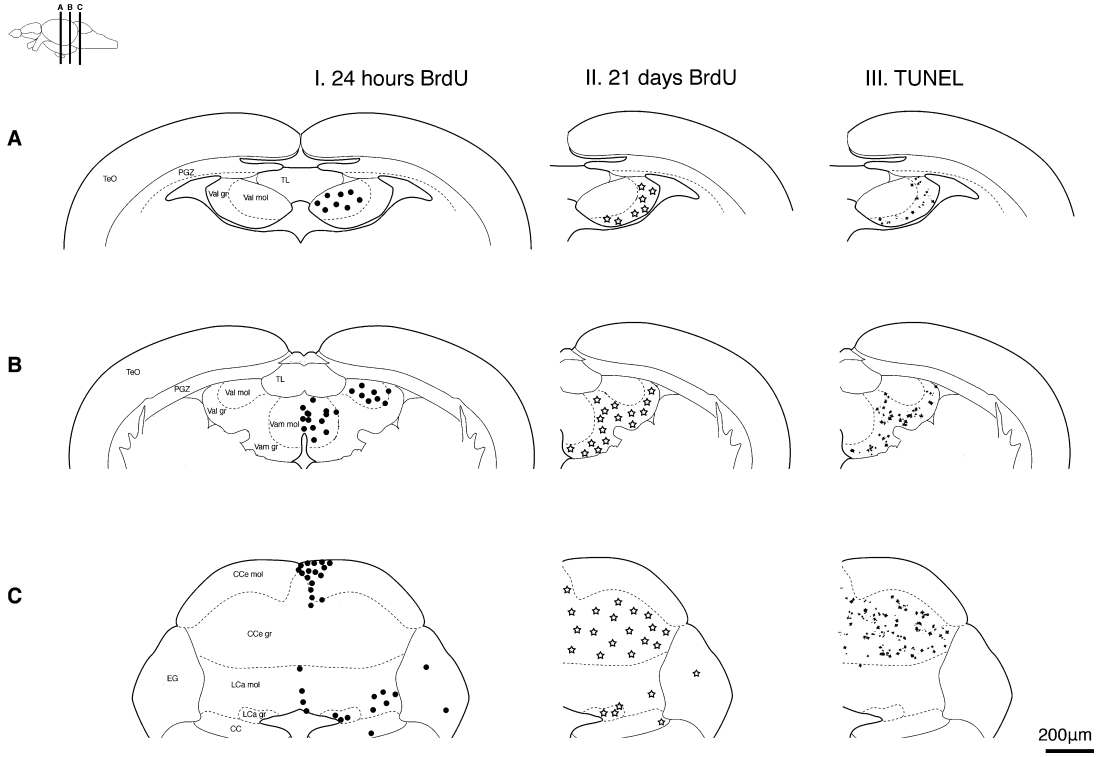

FIG. 2. (I and II) The distribution of newborn cells in the adult zebrafish cerebellum (I) 24 h and (II) 21 days post-BrdU administration. Each symbol (● or ☆) represents ~5–11 of the labelled cells observed in the tissue. (III) Distribution of apoptotic cells and bodies in cerebellum of adult zebrafish. A,B and C indicate the position of the coronal sections shown in the lateral view of the brain in the top right corner, A anterior; C posterior. Symbols represent the density of labelled cell fractions observed in the sections.

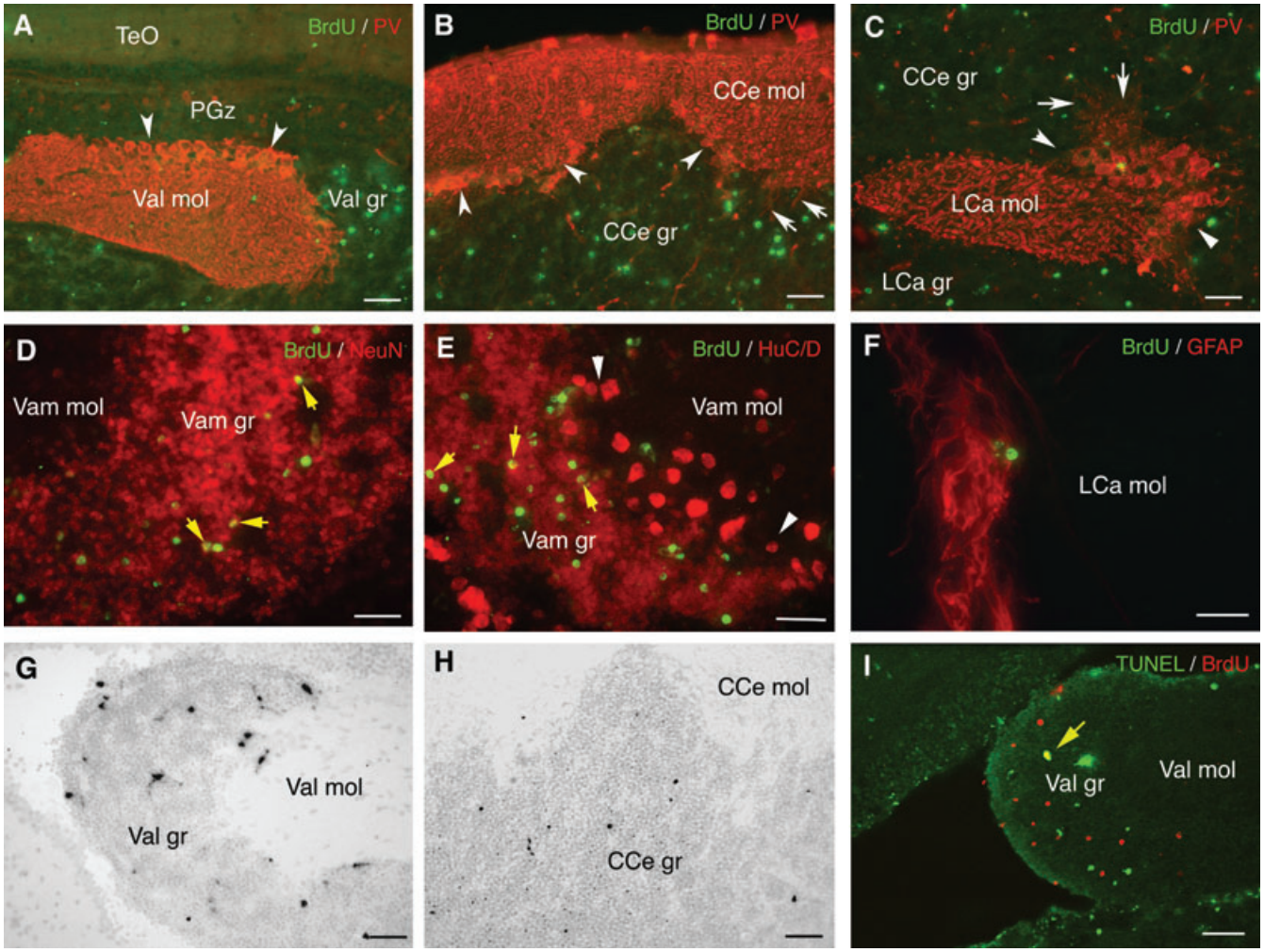

FIG. 3. (A–E) Photomicrographs of transverse sections of the zebrafish cerebellum showing the distribution of newborn cells 21 days post-BrdU administration (green) with PV-positive neurons (red; A–C), with NeuN-positive neurons (red; D), and with HuC/D-positive neurons (red; E). (F) Double-labelling image for BrdU (21 days; green) and GFAP (red). (G and H) Photomicrographs of Va and CCe after TUNEL immunohistochemistry. (I) Photomicrograph of BrdU (21 days; red) and TUNEL (green) double-labelling immunofluorescence. Arrowheads indicate the Purkinje (ganglionic) cell layer. Arrows indicates the PV-positive axons of Purkinje cells. Yellow arrows indicate double-labelled cells. TeO, optic tectum; PGz, periventicular gray zone of optic tectum. Scale bar, 0.01 mm.

newborn cells were found in a chain-like formation (Fig. 1), possibly following a chain migration-like method (Lois *et al*., 1996).

Quantification of labelled cells was performed to estimate and compare the number of BrdU-positive profiles in adult male and female zebrafish within the cerebellar areas. In all cases, male individuals had higher numbers of newly born cells (Fig. 4). Comparison of the numbers of labelled cells in each layer between short- and long-term experiments showed statistically significant differences in Vam-mol ($F_{1,8} = 50.94$, $P << 0.05$), Vam-gr ($F_{1,8} = 34.25$, $P = 0.0001$), Val-mol ($F_{1,8} = 51.51$, $P = 0.0001$), Val-gr ($F_{1,8} = 88.089$, $P << 0.05$), CCe-mol ($F_{1,8} = 39.36$, $P << 0.05$) and CCe-gr ($F_{1,8} = 29.46$, $P = 0.0002$) in both male and female animals. Statistically significant differences between the sex groups (Fig. 5A) were found in CCe-mol ($F_{1,8} = 10.25$, $P = 0.0084$) and in LCa-gr ($F_{1,8} = 10.14$, $P = 0.011$) in the short-term survival group (24 h). In long-term survival experiments, there were significantly higher numbers of BrdU-positive cells in Vam-gr ($F_{1,8} = 8.075$, $P = 0.016$) in male than in female cerebellum (Fig. 5B).

The density of granule cells was estimated by quantification of the Nissl-labelled cells in the granule cell layer of different cerebellar compartments and was estimated, for males and females respectively, to be in Vam 30.25 ± 8 and 26.7 ± 8 per 0.001 mm^2, in LCa 25.83 ± 6 and 23.74 ± 8 per 0.001 mm^2 and in CCe 46.39 ± 7 and 32.41 ± 5 per 0.001 mm^2. Unpaired *t*-tests showed a statistically significant difference in CCe ($t_2 = 2.77$, $P = 0.015$), with males exhibiting a higher density of granule cells. This sex-specific difference in the density of granule cells in CCe-gr is well correlated with the sex-specific difference in the density of proliferating cells in CCe-mol (Fig. 5A).

In addition to the high proliferative activity, significant apoptotic activity in cerebellar areas was observed, mainly in the granule cell layer, the final destination of proliferating cells. The characteristic appearance of apoptotic bodies in single cells (Wyllie, 1997) was visualized by the TUNEL method. Specifically, a high number of TUNEL-labelled cells was present in the granular layer (Figs 2, IIIA–C, 3G–I and 6) of Val-gr, in Vam-gr and in CCe-gr. A few cells were present in Val-mol and Vam-mol. There were no sex-specific differences in the density of apoptotic figures in all cerebellar areas studied (Fig. 6). Double-labelling BrdU–TUNEL experiments showed that the majority of TUNEL-positive figures were not BrdU-labelled cells (Fig. 3I), indicating that apoptosis occurred in a different cell

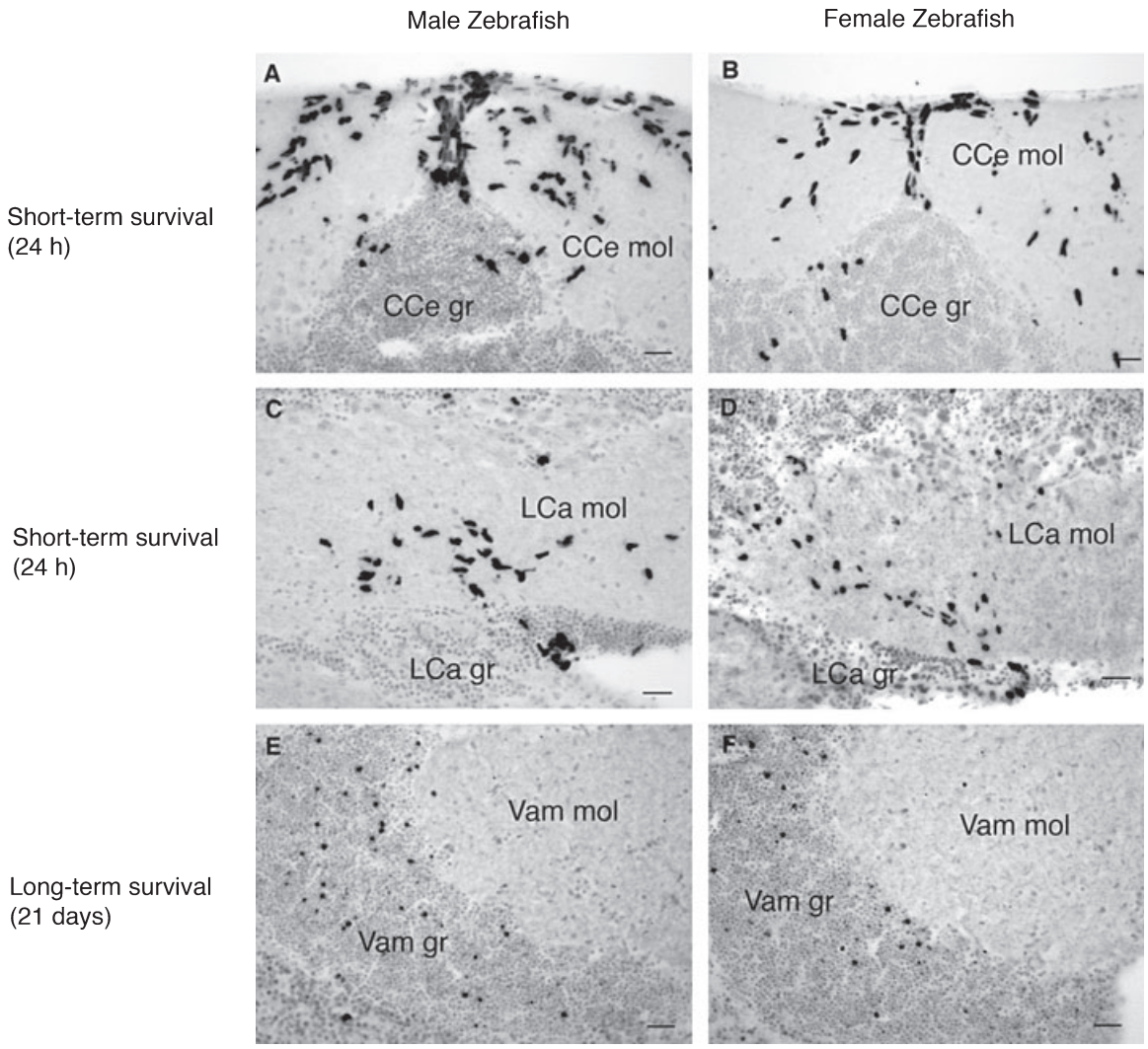

FIG. 4. Transverse sections of adult zebrafish cerebellum demonstrating differences in density of proliferating cells between sexes. (A and B) BrdU-positive cells 24 h post-BrdU administration (short-term survival) in (A) male and (B) female CCe. (C and D) Mitotic activity after 24 h post-BrdU administration (short-term survival) in (C) male and (D) female LCa. (E and F) Newborn cells in (E) male and (F) female Vam 21 days after (long-term survival) BrdU injection. Scale bar, 0.01 mm.

population within the granule cell layer from that of BrdU-retaining cells.

Discussion

The cerebellum is a highly conserved structure, composed of antero-posterior and mediolateral modular compartments in which distinct components of motor behaviour are presumably stored and modified when mastering motor tasks or acquiring reflexes (Ito *et al*., 1982; De Zeeuw *et al*., 1994; Herrup & Kuemerle, 1997; Ito, 2000; Han *et al*., 2006). However, the observed impressive rate of cell proliferation is unique to the cerebellum of adult teleost fish among all vertebrates. In agreement with previous studies in adult teleost fish, granule cells are produced in the molecular layer (Zupanc & Horschke, 1995; Zikopoulos *et al*., 2000; Ekström *et al*., 2001; Zupanc, 2001; Zupanc *et al*., 2005; Grandel *et al*., 2006) with no obvious comparable situation in amniotes. However, in the early stages of postembryonic life of zebrafish, a transient proliferative superficial secondary matrix very similar to the external granular layer in birds (Hallonet *et al*., 1990; Alvarez Otero *et al*., 1993; Stamatakis *et al*., 2004) and mammals (Altman, 1972; Rakic, 1973; Gao & Hatten, 1994; Alder *et al*., 1996; Jankovski *et al*., 1996; Zhang & Goldman, 1996) gives rise to inward-migrating neuroblasts that develop into granular cells (Wullimann & Knipp, 2000).

Specifically, in the adult male and female zebrafish brain, significant mitotic activity was observed in the molecular layer of medial and lateral divisions of Va (which is thought to be phylogenetically new as it is restricted to actinopterygians: Meek & Nieuwenhuys, 1998), CCe-mol (thought to be homologous with the vermis of amniotic vertebrates: Ito, 1978) and LCa-gr. Low mitotic activity was found in Val-gr and Vam-gr. Quantification of the BrdU-positive cell densities clearly showed that Val-mol exhibited the higher mitotic activity followed by Vam-mol, CCe-mol and LCa-gr, in both male and female zebrafish. These short-survival experiments probably represent less than the cell cycle length based on studies in the developing murine neocortex (Nowakowski *et al*., 2002). It is established that the number of cells labelled by BrdU changes dramatically with time, as a function of the number of proliferating cells in the population, the length of the S-phase, the length of the cell cycle and cell death (Hayes & Nowakowski, 2002). The length of the cell cycle is known to

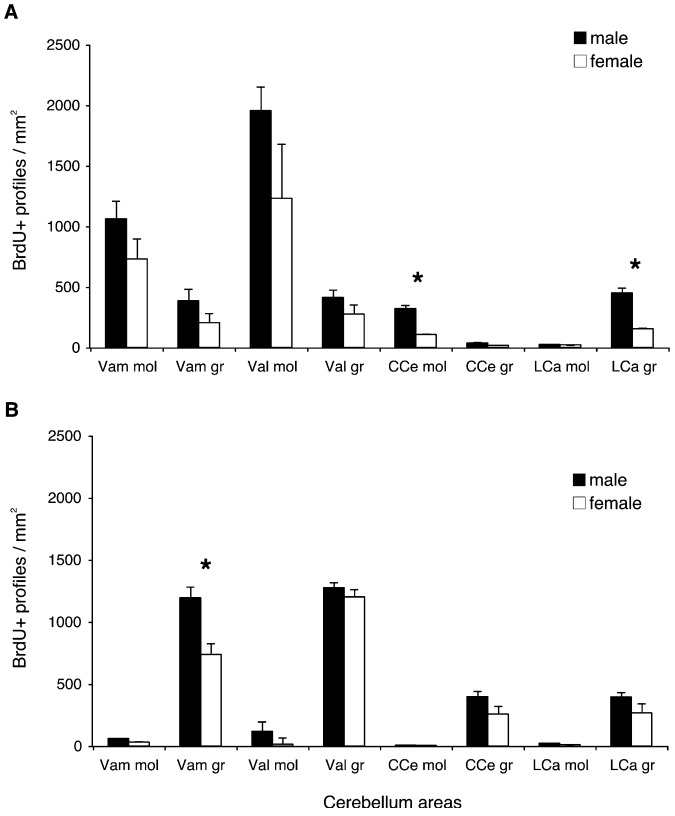

FIG. 5. Number of BrdU-positive cell profiles (mean ± SEM) per unit area (1 mm^2 of 20-μm-thick sections) in Vam-mol, Vam-gr, Val-mol, Val-gr, CCe-mol, CCe-gr, LCa-mol and LCa-gr of the adult zebrafish cerebellum, measured in male and female individuals, (A) 24 h and (B) 21 days after BrdU administration. *$P < 0.05$ (Statgraphics Plus 5.0) between the sex groups.

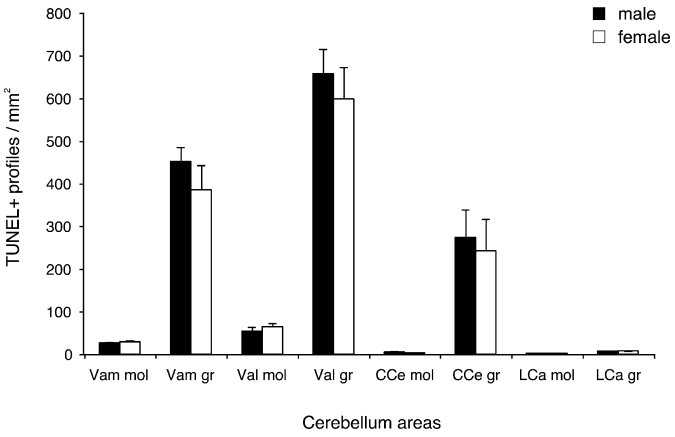

FIG. 6. Number of apoptotic cell profiles (mean ± SEM) per unit area (1 mm^2 of 20-μm-thick sections) in Vam-mol, Vam-gr, Val-mol, Val-gr, CCe-mol, CCe-gr, LCa-mol and LCa-gr of the adult zebrafish cerebellum, measured in male and female individuals.

rapidly increase during development and in early zebrafish CNS (24–28 hours post fertilization) it already amounts to 10 h (Li *et al*., 2000). Taken together with the BrdU availability for ∼4 h (Zupanc & Horschke, 1995), the survival period of 1 day most probably represents only one cell cycle in adult zebrafish cerebellum. For the long-term survival groups, however, proliferating cells labelled at 20 h will produce labelled daughter cells if they continue to divide, as shown in mammals (Hayes & Nowakowski, 2002). Therefore, one must keep in mind that long-survival experiments integrate proliferation, migration and cell death events. In long survival, progenitor cells generated in the molecular layer either remained in the precursor pool or entered the postmitotic population and migrated from their origin site to the corresponding granular layer. It is suggested that the majority of newborn cells (∼ 80%) complete their migration after 10 days of survival (Zupanc *et al*., 2005). While this general pattern of proliferating cells in adult zebrafish cerebellum is in good agreement with that previously described (Zupanc *et al*., 2005), the present study adds important information on cell proliferation and migration at intermediate survival times (24 h and 21 days), in addition to the determination of sex differences. In agreement, 21 days after BrdU administration the vast majority of the newborn cells were found in the respective granular layers of Va and CCe, while only a few labelled cells remained in their proliferation site in the molecular layer. Comparisons of the densities of labelled cells in short vs. long survival times in both molecular and associated granular layers of each area did not show significant increases in the number of cells, suggesting no further division of labelled cells. In the present study in adult zebrafish cerebellum, 24 h post-BrdU, when most labelled cells were in the process of migration, only a few newborn cells were found in close apposition to GFAP-positive glial fibres, while the majority seemed to follow a chain-like migration, not guided by glial processes. In contrast, cerebellar lesion studies in gymnotiforms suggest that the new cells are guided to the lesion site by radial glial fibres (Clint & Zupanc, 2001; Zupanc & Clint, 2003).

In long-term survival experiments there were no BrdU–PV double-labelled cells, clearly showing that no Purkinje cells are produced in adult cerebellum. The vast majority of adult Purkinje cells exhibited strong characteristic PV staining in all zebrafish cerebellar regions. In agreement, Porteros *et al*. (1998) have reported that their anti-PV antibody also recognised Purkinje cells and that they have observed eurydendroid cells surrounded by immunopositive Purkinje synapses. Eurydendroid cells in the Purkinje cell layer are large cells, output neurons for the cerebellum, as teleosts do not have the deep nuclei that are present in amniote vertebrates. While PV staining differed between eurydendroid and Purkinje cells, the latter did not exhibit any staining differences among cerebellar regions to support a possible functional compartmentalisation of zebrafish cerebellum, as has been suggested by the zonal distribution of several subtypes of Purkinje cells with different dendritic patterns, e.g. planar in Vam but arbor-shaped in the caudal CCe (Miyamura & Nakayasu, 2001), as well as by the pattern of acetylcholinesterase activity of Purkinje cells (Clemente *et al*., 2004).

In addition to possible Purkinje cell subpopulations, it has been suggested that granule cells show different populations. Fish granule cells use the excitatory neurotransmitter glutamate in their synapses with Purkinje cells (Somogyi *et al*., 1990), but only one subpopulation is thought to show acetylcholinesterase reactivity in the zebrafish (Clemente *et al*., 2004), although in the goldfish (Contestabile *et al*., 1977) and in the catfish (Contestabile *et al*., 1977) such differences in granule cell populations have not been reported. In the present study the vast majority of newborn cells were added and homogeneously distributed in the granular cell population, although a proportion of these BrdU-retaining cells were not labelled by the two neuronal markers used. The present findings indicated that a population of BrdU-labelled cells expressed the HuC/D neuronal phenotype, in agreement with a recent study (Grandel *et al*., 2006). In addition, most granule cells were found to express the postmitotic neuronal marker NeuN, in accordance with a study in goldfish (King *et al*., 2004). Further studies are necessary to characterize these newly adult-generated granule cells

and to determine whether they represent a specific subpopulation of the zebrafish granule cells.

Apoptosis and cell addition in adult zebrafish cerebellum

The molecular basis of apoptosis during zebrafish development has been described by Yamashita (2003). In adult zebrafish, apoptosis has been previously studied in the olfactory bulbs following the removal of the olfactory organ (Vankirk & Byrd, 2003). In the present study, TUNEL-positive cell profiles were mainly included in the granule cell layer of Va and CCe cerebellum, while the molecular layer, the site of cell proliferation, showed low apoptotic activity, in agreement with previous studies in adult gymnotiform cerebellum (Soutschek & Zupanc, 1996). In contrast, studies in mammals have reported that apoptosis is prominent in the proliferating neuroepithelium of the developing rat cerebral cortex and that it is related to the progression of the cell cycle (Thomaidou *et al*., 1997). Similarly, in the early postnatal mammalian cerebellum, apoptosis is thought to regulate the size of the rapidly expanding population of premigratory cells in the external granular layer, while the incidence of cell death of postmitotic granule cells in the internal granular layer is reported as increasing with age and appears to be linked to the formation of the mature synaptic circuitry of the developing cerebellar cortex (Lossi *et al*., 2002; Lossi & Merighi, 2003). In the adult zebrafish cerebellum, apoptosis was correlated with the postmitotic granule cells and not the precursor cells, supporting the hypothesis that apoptosis in adults has a role in the regulation of the cell density of postmitotic differentiated areas. In agreement, it is suggested that apoptotic cells appear in proliferating zones in early in development while at later stages they are found in postmitotic differentiated areas in the brown trout, *Salmo trutta fario* (Candal *et al*., 2005).

Male and female animals showed similar densities of apoptotic figures in the present study and the relatively high density of apoptotic figures found in the granular layer of zebrafish cerebellum is probably related to the increased addition of new cells, suggesting that the occurrence of cell death is counteracting it. Indeed, when different cerebellar regions are compared, the proliferation rate and the addition of newborn cells parallels the rate of apoptotic cell death; that is, areas with higher addition of newborn cells exhibit higher apoptotic rates, e.g. in Val-gr cell death density is higher than in CCe-gr, as is the density of newborn cells in Val-mol compared to CCe-mol. Interestingly, cell death was low in both LCa-mol and LCa-gr, and did not show migration of newborn cells. In agreement, programmed cell death is correlated with high proliferative activity in adult gymnotiform fish cerebellum, *Apteronotus leptorhynchus* (Soutschek & Zupanc, 1996; Zupanc *et al*., 1998). The observed density of apoptotic cells in the granular layer of adult zebrafish cerebellum is higher than that reported in other regions of the adult brown trout teleost brain (Candal *et al*., 2005) but is similar to that of cells which have been deafferented for 1 h in the olfactory bulb (Vankirk & Byrd, 2003).

This phenomenon of the close relation between cell birth and cell death is widely accepted. In the gymnotiform fish cerebellum (Soutschek & Zupanc, 1996) is has been suggested that the newly generated cells die, as a possible mechanism for eliminating cells that do not make the proper connections; this has been proposed for the establishment of connections during development (Oppenheim, 1991). Alternatively, in the case of older cells dying, is suggested that these cells are replaced by newborn ones. Lesion studies in adult brain suggest the replacement with newly generated cells of cells lost because of injury (Zupanc & Ott, 1999). In the present study, double-labelling experiments revealed that only in a few cases was there colocalization of TUNEL and BrdU at the specific time points studied. This evidence clearly indicates that the majority of granule cells undergoing apoptosis were not generated at the time of BrdU injection. Therefore, the incidence of apoptosis mainly occurred in a different cell population than that of the BrdU-retaining cells, favouring the hypothesis of the replacement of older cells by new ones. However, the present study cannot conclude whether old or new cells die to elucidate a possible plasticity mechanism in the adult zebrafish cerebellum. Double-labelling experiments at serial time courses are required for this characterisation of the apoptotic cells. However, it is interesting that the density of the proliferating progenitor cells matched the sum of BrdU-retaining cells in the long-survival animals with the densities of apoptotic cells (both in proliferation and final destination sites). This evidence, in addition to the fact that cell death is minimum in LCa where cells do not migrate long distances but remain in their proliferation site, favours the hypothesis of the apoptosis of the generated cells during their migration and establishment of proper connections.

Sex differences in cell proliferation in adult zebrafish cerebellum

Brain mitotic activity, using the BrdU immunoassaying method, has been extensively used to study sex differences in mammals (Galea & McEwen, 1999; Falconer & Galea, 2003; Abizaid *et al*., 2004), birds (Nikolakopoulou *et al*., 2006) and fish (Zikopoulos *et al*., 2000, 2001). Quantification of BrdU-labelled cells in adult zebrafish revealed significant differences in both proliferation and survival of newborn cells between male and female cerebellar areas. Specifically, in CCe-mol and LCa-gr, a higher rate of proliferation was found in males. In the case of CCe-mol, cell production resulted sex-specific differences in the granule cell density in CCe-gr. In support of our evidence on sex-specific cell proliferation and cell survival, Gelinas & Callard (1997) demonstrated neurons with immunoreactive androgen receptors in the molecular layer of the goldfish (*Carassius auratus*) Va. In addition, is known that adult teleost fish express extraordinarily high levels of brain aromatase activity when compared to the brain of mammals (Callard *et al*., 1990) and, in the midshipman, these high levels of aromatase expression are localized in glia and not neurons (Forlano *et al*., 2001). Whether neuronal or glial, the high levels of aromatase in the adult teleost brain may provide large quantities of oestrogenic compounds that are thought to induce continuous cell proliferation (Gelinas *et al*., 1998) and may therefore influence mitotic activity in a sex-specific manner. In agreement, in mammals, oestrogen is widely documented as acting as a growth-stimulating agent, modulating apoptotic cell death and affecting migration of neuroblasts from the subventricular layer, mostly seen in distinct brain nuclei that are oestrogen-sensitive and become sexually dimorphic in adults (for review, see Beyer, 1999; Wise *et al*., 2001).

Most of the studies on steroid-specific brain areas are focused on forebrain, and zebrafish brain is reported as expressing high aromatase activity, P450 aromB being the predominant isoform, in both males and females, with no sex difference (Sawyer *et al*., 2006). At present, there is not enough detailed anatomical evidence to determine whether zebrafish cerebellum is a steroid-sensitive area, although it exhibited sex-specific adult cell proliferation. Whether this sex-specific difference in cell proliferation rate is directly depended on sex steroids remains to be elucidated. However, this is not always the case as, in a study in the oyster toadfish (Fine *et al*., 1996), steroid sensitive areas did not always exhibit sex-specific differences; further, regions showing sex-specific differences did not necessarily exhibit sex differences in steroid levels.

Functional implications of sex differences in cell generation and addition in adult zebrafish cerebellum

The capacity of neurogenesis and plasticity in adult fish resembles properties seen in embryonic mammalian and avian brain (Levine *et al*., 1994). The teleostean brain continues to grow throughout life and retains a remarkable potential for neuroregeneration (Yoon, 1975; Raymond & Easter, 1983; Stuermer, 1988). The continual addition of new cells may enable structural changes centrally that may be required for long-term changes in behaviour (for review, see Zupanc, 1999). The addition of new cells is most significant in the adult cerebellar structures, suggesting the functional plasticity of cerebellum. Moreover, this significant addition of new cells showed sex-specific differences, further providing evidence for the sexual plasticity of this area and a possible developmental mechanism with many implications, including motor learning plasticity.

While classic neurobiology views the cerebellum as an essential part of the motor control system, a growing amount of evidence implicats mammalian and human cerebellum in a wide spectrum of cognitive and emotional functions: for example, additional cerebellar functions include spatial learning (Thompson *et al*., 1997), timing, sensory acquisition and attention (Allen *et al*., 1997), problem-solving, error detection and language (Leiner *et al*., 1993), emotions (Supple & Kapp, 1993) and others. Interestingly, an early lesion study related the fish cerebellum to learning and memory processes such as motor conditioning and avoidance learning (Aronson & Herberman, 1960). The contribution of the teleost fish cerebellum to classical fear heart rate conditioning has been investigated recently in goldfish (Rodriguez *et al*., 2005), suggesting a role of cerebellum in emotional learning in addition to spatial learning (Rodriguez *et al*., 2005). Therefore, the mammalian and the teleostean cerebellum might share a striking functional similarity in the classical conditioning of simple motor reflexes, in emotional fear heart rate conditioning and in spatial cognition. In view of this suggested role of teleost cerebellum in spatial and emotional learning, it might be challenging to speculate on a possible relation between the integration of newly generated cells and memory in the cerebellum. Moreover, sex steroid hormones are implicated in the cognitive processes of the adult brain and new granule cells in the adult dentate gyrus of mammalian hippocampus are known to be incorporated into the existing circuitry (Gould *et al*., 2001) in a sexually dimorphic manner (Tanapat *et al*., 1999; Falconer & Galea, 2003, 2006). Such sex-specific addition of newly born cells is also the case for granule cells in the adult teleost cerebellum, further supporting the possible relationship of cell proliferation and adult sexual plasticity and behaviour, correlating higher proliferation rates with sex-specific motor behaviour of males. A sexually differentiated neurochemical control of the cerebellum could therefore play a role in the organization of male locomotor activity.

Acknowledgements

Authors thank Professor M. Kentouri, Aquaculture Laboratory (Biology Department, University of Crete) for providing the initial stock of zebrafish and Dr M. F. Wullimann for important discussions on evolutionary concepts of zebrafish cerebellum. This project was partly supported by EU grant EU-QLRT-1999–31629 and is co-funded by the European Social Fund and national resources.

Abbreviations

BrdU, bromodeoxyuridine; CC, crista cerebellaris; CCe, corpus cerebelli; EG, eminentia granularis; GFAP, glial fibrillary acidic protein; -gr, granular cell layer; LCa, caudal lobe of cerebellum; -mol, molecular cell layer; NeuN, neuronal nuclei; PBS, phosphate-buffered saline; PGZ, periventicular gray zone of optic tectum; PV, parvalbumin; RT, room temperature; TdT, terminal deoxynucleotidyl transferase; TeO, optic tectum; TUNEL, TdT-mediated dUTP nick-end labelling; Va, valvula cerebelli; Val, lateral division of the Va; Vam, medial division of the Va.

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