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

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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 medio-dorsal 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; Räsänen 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.
Sex differences in zebrafish cerebellum

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 H2O2 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% H2O2 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)H2O, or 0.5% Cresyl Violet in ddH2O) 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 immunohistofluorescence

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 45Ca-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 ependymal 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

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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 antirat Alexa Fluor 568 (Molecular probes; diluted 1 : 500 in 0.5% Triton X-100 in PBS; Molecular probes) 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 an 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 perform the TUNEL reaction. The sections were then incubated with an antidigoxigenin antibody which is conjugated to a fluorescein reporter molecule (ApopTag Kit, S7110; Chemicon). Additional BrdU–HuC/D antibodies were added to sections for 3 h at 10 °C (Accurate Chemical) for BrdU immunohistochemistry using a monoclonal mouse anti-BrdU (1 : 100; Becton Dickinson) and an antimouse Alexa Fluor 568 (1 : 500 in 0.5% Triton X-100 in PBS; 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 DSC-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 mm² 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-gmol 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-gmol, using the software VolumeJ. The arrows indicate the chain migration-like pattern of newly born cells. Scale bar, 0.1 mm.](image-url)
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 CCo-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 CCo (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 CCo 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.
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 \((F_{1,8} = 88.09, P < 0.05)\), 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 \((t_2 = 2.77, P = 0.015)\), 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 compartment and was estimated, for males and females respectively, to be in Vam 30.25 ± 8 and 26.7 ± 8 per 0.001 mm², in LCa 25.83 ± 6 and 23.74 ± 8 per 0.001 mm² and in CCe 46.39 ± 7 and 32.41 ± 5 per 0.001 mm². 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).
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; Zupane, 2001; Zupane 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. 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.](image-url)
administration. In male and female individuals, (A) 24 h and (B) 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 telocasts 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 C Ce 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 C Ce-gr, as is the density of newborn cells in Val-mol compared to C Ce-mol. Interestingly, cell death was low in both L Ca-mol and L Ca-gr, and did not show migration of newborn cells. In agreement, programmed cell death is correlated with high proliferative activity in adult gymnnotiform fish cerebellum, Apertorontus 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 bull 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 gymnnotiform fish cerebellum (Soutschek & Zupanc, 1996) it 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 L Ca 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 C Ce-mol and L Ca-gr, a higher rate of proliferation was found in males. In the case of C Ce-mol, cell production resulted sex-specific differences in the granule cell density in C Ce-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, it 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 dependent on sex steroids remains to be elucidated. However, this is not always the case as, in a study in the oyster toadfish (Fines 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.

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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 implicates mammalian and human cerebellum in a wide spectrum of cognitive and emotional functions: for example, additional cerebellar functions include spatial learning (Thompson et al., 2000), 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 (Arondson & 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.

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Abbreviations

BrdU, bromodeoxyuridine; CC, crista cerebellaris; CCE, corpus cerebelli; EG, eminenta 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, periventricular 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.

References


