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REVIEW

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# Neurogenesis and neuronal regeneration in the adult fish brain

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**Abstract** Fish are distinctive in their enormous potential to continuously produce new neurons in the adult brain, whereas in mammals adult neurogenesis is restricted to the olfactory bulb and the hippocampus. In fish new neurons are not only generated in structures homologous to those two regions, but also in dozens of other brain areas. In some regions of the fish brain, such as the optic tectum, the new cells remain near the proliferation zones in the course of their further development. In others, as in most subdivisions of the cerebellum, they migrate, often guided by radial glial fibers, to specific target areas. Approximately 50% of the young cells undergo apoptotic cell death, whereas the others survive for the rest of the fish's life. A large number of the surviving cells differentiate into neurons. Two key factors enabling highly efficient brain repair in fish after injuries involve the elimination of damaged cells by apoptosis (instead of necrosis, the dominant type of cell death in mammals) and the replacement of cells lost to injury by newly generated ones. Proteome analysis has suggested well over 100 proteins, including two dozen identified ones, to be involved in the individual steps of this phenomenon of neuronal regeneration.

**Keywords** Adult neurogenesis · Neuronal regeneration · Proteomics · *Apteronotus leptorhynchus* · Zebrafish

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**Abbreviations** *BrdU*: 5-Bromo-2′-deoxyuridine · *CP*/*PPn*: Central posterior/prepacemaker nucleus · *EOD*: Electric organ discharge · *GFAP*: Glial fibrillary acidic protein · *TUNEL*: Terminal deoxynucleotidyl-transferase-mediated dUTP-biotin nick end-labeling

## Introduction

Until a few decades ago, it was thought that the brain of vertebrates ceased to produce new neurons at, or shortly after, birth. This "no new neurons" dogma was challenged only in the 1960s when Joseph Altman of the Massachusetts Institute of Technology provided, by use of [$^3$H]thymidine autoradiography, evidence that new neurons are formed in the adult mammalian brain (Altman 1962, 1963, 1969a, b; Altman and Das 1965). Since then, this phenomenon, now commonly referred to as adult neurogenesis, has been demonstrated not only in a number of mammalian species (Mareš and Lodin 1974; Kaplan and Hinds 1977; Kaplan 1981; Bayer et al. 1982; Kaplan and Bell 1983; Corotto et al. 1993; Lois and Alvarez-Buylla 1993, 1994; Gould et al. 1997, 1998, 1999; Eriksson et al. 1998; Van Praag et al. 2002; Song et al. 2002; for reviews see Doetsch and Scharff 2001; Temple 2001; Gage 2002; Taupin and Gage 2002; Rakic 2002), but also in all other major vertebrate classes, including bony fishes (Johns 1977; Meyer 1978; Raymond and Easter 1983; Zupanc and Zupanc 1992; Zupanc and Horschke 1995; Zupanc et al. 1996, 2005; for reviews see Zupanc 1999a, 2001a), amphibians (Bernocchi et al 1990; Chetverukhin and Polenov 1993; Polenov and Chetverukhin 1993), reptiles (López-García et al. 1988; García-Verdugo et al. 1989; Pérez-Sánchez et al. 1989; Pérez-Cañellas and García-Verdugo 1996; for review see Font et al. 2001), and birds (Goldman and Nottebohm 1983; Paton and Nottebohm 1984; Burd and Nottebohm 1985; Paton et al. 1985; for review see Nottebohm 2002).

Despite the presumptive universal occurrence of adult neurogenesis within the vertebrate taxon, comparative analysis has revealed pronounced differences between "lower" and "higher" vertebrates. For example, whereas in mammals the generation of new neurons appears to be restricted to just two brain regions (see Cell proliferation–Olfactory bulb and Dorsal telencephalon, below), and the number of new neurons is extremely small compared to the total number of brain cells, in fish an enormous number of neurons is continuously produced in many areas of the intact brain. Furthermore, while replacement with new neurons of whole neuronal cells damaged through injury or neurodegenerative disease is usually impossible in the mammalian central nervous system (Goldman-Rakic 1980), fish exhibit an enormous potential for so-called neuronal regeneration after injury (Kirsche 1950; Botsch 1960; Kirsche and Kirsche 1961; Pflugfelder 1965; Bernstein 1968; Meyer et al. 1985; Zupanc et al. 1998; Zupanc and Ott 1999; for reviews see Waxman and Anderson 1986; Zupanc 1999a; 2001a).

The difference between fish and mammals in their potential for both adult neurogenesis and neuronal regeneration makes it extremely attractive to study fish. Identification of the cellular mechanisms that mediate adult neurogenesis and neuronal regeneration in fish will not only answer fundamental biological questions, but is also likely to provide important insights into the factors that limit these phenomena in the mammalian brain. Such an approach has, therefore, an enormous potential to define novel strategies for wound healing and treatment of neurodegenerative diseases.

The study of adult neurogenesis and neuronal regeneration in fish has enormously benefitted from the availability of the brown ghost knifefish (*Apteronotus leptorhynchus*; Gymnotiformes, Teleostei; Fig. 1) as a major model system (for reviews see Waxman and Anderson 1986; Zupanc 1999a, 2001a). Since this weakly electric fish has been intensively studied to reveal neural correlates of electric behaviors (for reviews see Zupanc and Maler 1997; Nelson and MacIver 1999; Turner and Maler 1999; Bastian 1999; Metzner 1999; Zakon et al. 2002; Zupanc 2002; Zupanc and Bullock 2005), the structure and function of many of its brain regions are better characterized than in any other teleost. As a result, *A. leptorhynchus* was the first teleostean species for which a complete, detailed brain atlas was available (Maler et al. 1991). This large body of morphological and physiological information has provided an excellent basis to analyze the pattern of postembryonic brain development and to relate the findings to possible functions. Moreover, *Apteronotus* is ideally suited to apply defined mechanical lesions to the brain (see "Neuronal regeneration—The lesion paradigm"), enabling investigators to expand the study of adult neurogenesis from the intact to the injured central nervous system.

**Fig. 1** The brown ghost knifefish (*Apteronotus leptorhynchus*)

## Cell proliferation

Quantitative analysis of the pattern of cell proliferation in the brain of *Apteronotus* has been based on the incorporation of the thymidine analog 5-bromo-2′-deoxyuridine (BrdU) into newly synthesized DNA during the S-phase of mitosis, followed by visualization of BrdU-labeled cells through anti-BrdU immunohistochemistry (Gratzner et al. 1975; Gratzner 1982). Such studies have revealed an enormous number of mitotic cells in many brain regions (Figs. 2, 3). On average, during any 2-h period, approximately 100,000 cells enter the S-phase of mitosis in the whole brain (Zupanc and Horschke 1995). This corresponds to roughly 0.2% of the total number of cells in the adult brain of *Apteronotus*. Approximately 25% of these new cells are generated in the telencephalon, diencephalon, mesencephalon, and rhombencephalon. The remaining 75% originate from the cerebellum.

In comparison to the enormous mitotic activity in the adult fish brain, the rate of cell proliferation in the adult mammalian brain is at least one, if not two, orders of magnitude lower. Louis and Alvarez-Buylla (1994) have estimated that, in adult mice, approximately 30,000 cells a day are formed in the subventricular zone, one of the two sites of ongoing neurogenesis in the adult mammalian brain. This number corresponds to 0.03% of the estimated 110 million cells (Williams 2000) in the whole brain of adult mice. In the dentate gyrus of the hippocampus—the second site where neurogenesis continues into adulthood in the mammalian brain—the production of only 9,000 cells a day has been found in the adult rat (Cameron and McKay 2001). The latter number corresponds to 0.003% of the approximately 330 million cells (Herculano-Houzel and Lent 2005) in the whole brain of the adult rat.

In the adult brain of *Apteronotus* and other teleosts, the vast majority of the mitotic cells are found, at high concentrations, in small, well-defined areas of the brain ("proliferation zones"), as they are in mammals. Many of these zones are situated at or near the surfaces of ventricles or related systems. Although other zones, particularly in the cerebellum, are located in regions

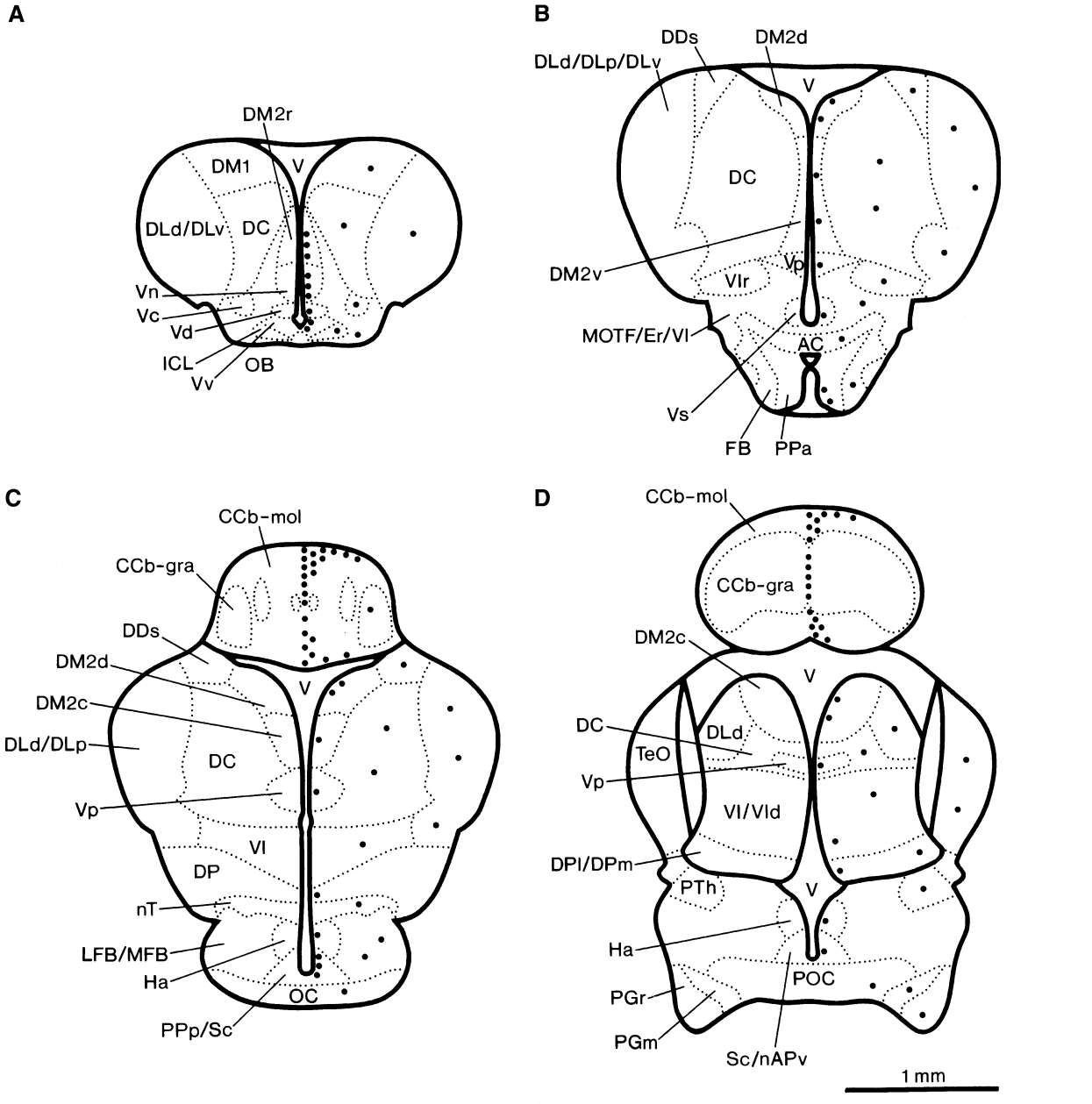



**Fig. 2** Proliferation zones in the telencephalon of adult *A. leptorhynchus*. The charts of transverse sections roughly correspond to levels 37 (**a**), 30 (**b**), 28 (**c**), and 26 (**d**) of the brain atlas of *A. leptorhynchus* (Maler et al. 1991). Proliferation zones, as revealed by incorporation of BrdU into replicating DNA and subsequent anti-BrdU immunohistochemistry, are indicated by *black dots*. *AC* anterior commissure; *CCb* corpus cerebelli; *DC* central division of the dorsal forebrain; *DDs* superficial subdivision of dorsal division of the dorsal forebrain; *DLd* dorsolateral telencephalon, dorsal subdivision; *DLp* dorsolateral telencephalon, posterior subdivision; *DLv* dorsolateral telencephalon, ventral subdivision; *DM1* dorsomedial telencephalon, subdivision 1; *DM2c* dorsomedial telencephalon, subdivision 2, caudal; *DM2d* dorsomedial telencephalon, subdivision 2, dorsal; *DM2r* dorsomedial telencephalon, subdivision 2, rostral; *DM2v* dorsomedial telencephalon, subdivision 2, ventral; *DP* dorsal posterior telencephalon; *DPl* lateral subdivision of caudal dorsal posterior telencephalon; *Er* rostral entopeduncular nucleus; *FB* forebrain bundle; *gra* granule cell layer; *Ha* hypothalamus anterioris; *ICL* internal cell layer of olfactory bulb; *LFB* lateral forebrain bundle; *MFB* medial forebrain bundle; *mol* molecular layer; *MOTF* medial olfactory terminal field; *nAPv* nucleus anterior periventricularis; *nT* nucleus taenia; *OB* olfactory bulb; *OC* optic chiasma; *PGm* preglomerular nucleus, medial subdivision; *PGr* preglomerular nucleus, rostral subdivision; *POC* postoptic commissure; *PPa* nucleus preopticus periventricularis, anterior subdivision; *PPp* nucleus preopticus periventricularis, posterior subdivision; *PTh* nucleus prethalamicus; *Sc* suprachiasmatic nucleus; *TeO* optic tectum; *V* ventricle; *Vc* ventral telencephalon, central subdivision; *Vd* ventral telencephalon, dorsal subdivision; *Vl* ventral telencephalon, intermediate subdivision; *Vld* ventral telencephalon, intermediate dorsal subdivision; *VIr* ventral telencephalon, intermediate rostral subdivision; *Vl* ventral telencephalon, lateral subdivision; *Vn* ventral telencephalon, nother subdivision; *Vp* ventral telencephalon, posterior subdivision; *Vs* ventral telencephalon, supracommissural subdivision; *Vv* ventral telencephalon, ventral subdivision (from Zupanc and Horschke 1995)

distant from any ventricle (see "Cell proliferation—Cerebellum", below), many are derived from areas located at ventricular surfaces during embryonic stages of development. Then, as a result of the everted development of the fish brain during embryogenesis, the associated ventricular lumina are obliterated and translocated (Powels 1978a, b). In *Apteronotus*, several dozens of proliferation zones have been identified in the adult brain (Zupanc and Horschke 1995). A similar large number has been found in two other teleostean species in which a detailed mapping of such zones has been performed—the three-spined stickleback (Ekström et al. 2001) and the zebrafish (Zupanc et al. 2005).

In the following subsections, I will describe and discuss the pattern of cell proliferation in five regions of the teleostean brain that are of special interest from a comparative and/or functional point of view: olfactory bulb, dorsal telencephalon, optic tectum, central posterior/prepacemaker nucleus, and cerebellum.

## Olfactory bulb

Quantitative analysis of BrdU labeling has shown that approximately 0.2% of all mitotic cells in the adult brain of *Apteronotus* are found in the olfactory bulb. Most of these cells are spread over the external (glomerular) layer, whereas only a few mitotic cells are located in the internal cell layer (Zupanc and Horschke 1995). Similar results have been obtained in other teleosts, including goldfish, Mediterranean barbel, carp, rainbow trout, and zebrafish (Alonso et al. 1989; Byrd and Brunjes 2001; Zupanc et al. 2005). In the latter species, double-labeling experiments have revealed expression of the neuron-specific protein Hu by BrdU-labeled cells after longer survival times, indicating neuronal differentiation of the new cells (Byrd and Brunjes 2001; Zupanc et al. 2005).

Despite the low number of cells produced, neurogenesis in the olfactory bulb of teleosts is interesting because the bulb is one of the two brain regions in which adult neurogenesis has been consistently demonstrated in mammals. Investigations in the latter taxonomic class have shown that progenitor cells of these neurons undergo cell division in the anterior part of the subventricular zone surrounding the lateral ventricles. From this proliferation zone, the neuroblasts migrate, as chains of closely apposed cells, over several millimeters along a specific pathway—the rostral migratory stream—to the olfactory bulb, where they differentiate into interneurons (Altman 1969b; Luskin 1993; Lois and Alvarez-Buylla 1994; Lois et al. 1996; Pencea et al. 2001).

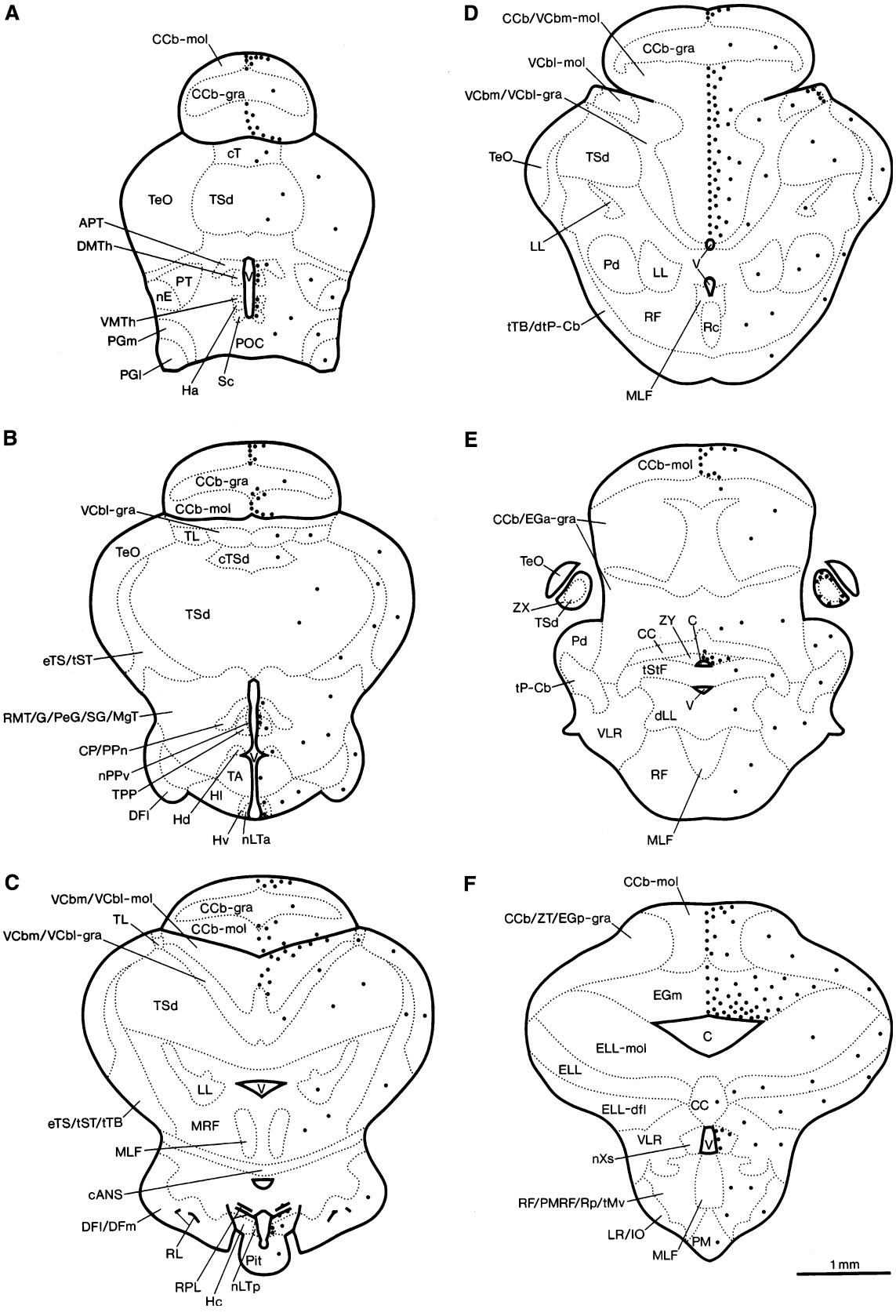
## Dorsal telencephalon

Among all telencephalic areas, the number of mitotic cells is largest in the dorsal, ventral, and posterior

**Fig. 3** Proliferation zones in the diencephalon, mesencephalon, and rhombencephalon of adult *A. leptorhynchus*. The charts of transverse sections roughly correspond to levels 23 (**a**), 17 (**b**), 13 (**c**), 7 (**d**), 2 (**e**), and −8 (**f**) of the brain atlas of *A. leptorhynchus* (Maler et al. 1991). Proliferation zones were revealed through labeling of mitotic cells with BrdU and are indicated by *black dots*. *C* cerebello-medullary cistern; *cANS* commissura ansulata; *CC* crista cerebellaris; *CCb* corpus cerebelli; *CP* central posterior nucleus; *cT* tectal commissure; *cTSd* commissure of the torus semicircularis dorsalis; *dfl* deep fiber layer of ELL; *DFl* nucleus diffusus lateralis of the inferior lobe; *DFm* nucleus diffusus medialis of the inferior lobe; *dLL* decussation of the lateral lemniscus; *dtP-Cb* decussation of tractus praeminentialis cerebellaris; *EGa* eminentia granularis pars anterior; *EGm* eminentia granularis pars medialis; *EGp* eminentia granularis pars posterior; *ELL* electrosensory lateral line lobe; *eTS* torus semicircularis efferents; *G* glomerular nucleus; *gra* granule cell layer; *Ha* hypothalamus anterioris; *Hc* hypothalamus caudalis; *Hd* hypothalamus dorsalis; *Hl* hypothalamus lateralis; *Hv* hypothalamus ventralis; *LL* lateral lemniscus; *LR* lateral reticular nucleus; *MgT* magnocellular tegmental nucleus; *MLF* medial longitudinal fasciculus; *mol* molecular layer; *MRF* mesencephalic reticular formation; *nE* nucleus electrosensorius; *nLTa* nucleus tuberis lateralis pars anterior; *nLTp* nucleus tuberis lateralis pars posterior; *nPPv* nucleus posterioris periventricularis; *nXs* vagal sensory nucleus; *Pd* nucleus praeminentialis dorsalis; *PeG* periglomerular nucleus; *PGl* preglomerular nucleus, lateral subdivision; *PGm* preglomerular nucleus, medial subdivision; *Pit* pituitary; *PM* pacemaker nucleus; *PMRF* paramedian reticular formation; *POC* postoptic commissure; *PPn* prepacemaker nucleus; *PT* pretectal nucleus; *Rc* nucleus raphé centralis; *RF* reticular formation; *RL* recessus lateralis; *RMT* rostral mesencephalic tegmental nucleus; *Rp* nucleus raphé posterioris; *RPL* recessus posterioris pars lateralis; *Sc* suprachiasmatic nucleus; *SG* subglomerular nucleus; *TA* nucleus tuberis anterior; *TeO* optic tectum; *TL* torus longitudinalis; *tMv* ventral medullary tract; *tP-Cb* tractus praeminentialis cerebellaris; *TPP* periventricular nucleus of the posterior tuberculum; *TSd* torus semicircularis, dorsal subdivision; *tST* subtectal tract; *tStF* tractus stratum fibrosum; *tTB* tectobulbar tract; *V* ventricle; *VCbl* valvula cerebelli pars lateralis; *VCbm* valvula cerebelli pars medialis; *VLR* ventrolateral rhombencephalon; *VMTh* ventromedial thalamus; *ZX* zone X; *ZY* zone Y (from Zupanc and Horschke 1995)

subdivisions of the dorsolateral telencephalon of *Apteronotus* (Zupanc and Horschke 1995). A similar concentration of proliferating cells has been reported for two other teleostean species, the three-spined stickleback (Ekström et al. 2001) and the zebrafish (Zupanc et al. 2005). These results are remarkable, since part of this brain region, commonly referred to as the lateral pallium, is thought to be homologous to the medial pallium or hippocampus of amniotes. In mammals, the hippocampus is the second brain region in which adult neurogenesis continues into adult stages of development (Altman and Das 1965; Kaplan and Bell 1984; Eriksson et al. 1998; Gould et al. 1999; Kornack and Rakic 1999; Seri et al. 2001). Similarly, neurogenesis has been found in the adult hippocampus of reptiles (López-García et al. 1988) and birds (Barnea and Nottebohm 1994).

Based on the taxonomic diversity of *A. leptorhynchus*, the three-spined stickleback, and the zebrafish, it appears reasonable to assume that the proliferation of cells in that portion of the dorsal telencephalon which is presumably homologous to the hippocampus is a conserved feature of teleosts. Evidence of neuronal




differentiation of the cells generated in the dorsal telencephalon has been obtained through double-labeling experiments in zebrafish (Zupanc et al. 2005). In the latter study, BrdU labeling was combined with immunohistochemistry against the neuron-specific marker protein Hu at post-BrdU administration survival times

of 9 months. These experiments have identified a large number of double-labeled cells in the region believed to be homologous to the mammalian hippocampus.

## Optic tectum

Analysis of the pattern of cell proliferation has shown that in the optic tectum of teleost fish new cells are generated predominantly in a specific proliferation zone at the caudal pole. Besides *A. leptorhynchus* (Zupanc and Horschke 1995), the list of species includes zebrafish (Marcus et al. 1999; Zupanc et al. 2005), goldfish (Meyer 1978; Raymond and Easter 1983), gilthead sea bream (Zikopoulos et al. 2000), and stickleback (Ekström et al. 2001). The similarity in the pattern of cell proliferation among these rather distantly related species suggests that the proliferation zone in the caudal tectum is a widespread, and probably universal, source of germinal cells in the adult brain of teleosts.

In both zebrafish and *Apteronotus* (Zupanc et al. 2005; G.K.H. Zupanc, unpublished observations), examination of sections through the optic tectum taken after various post-BrdU survival times has indicated that the vast majority of the new cells continue to reside in the proliferation zone of the caudal tectum. Similar results have been obtained in goldfish by labeling of new cells with tritiated thymidine and examination of autoradiographs after various postadministration survival times (Meyer 1978; Raymond and Easter 1983). Taken together, these results suggest that the optic tectum grows primarily from its caudal end.

Interestingly, the presence of the proliferation zone in the caudal optic tectum appears to be independent of whether or not sensory input is dominated by the visual modality in the various species. Whereas in zebrafish, goldfish, guppy, stickleback, and gilthead sea bream visual cues play a predominant role in the context of many behaviors, *Apteronotus* relies to a large extent on electric input for orientation and communication. It is unknown whether the differences in the amount of input received by the optic tectum from the eye lead to a quantitative difference in the number of new cells produced during adulthood. This possibility is worth examining because the optic tectum of *Apteronotus*, when compared to the rest of the brain, is significantly smaller than in more visually guided species, such as goldfish and zebrafish.

## Central posterior/prepacemaker nucleus

The central posterior/prepacemaker nucleus (CP/PPn) of gymnotiform fish is a bilateral cluster of approximately 10,000 neurons in the dorsal thalamus, stretching from the wall of the third ventricle approximately 500 μm in the ventrolateral direction (Zupanc and Heiligenberg 1992; Stroh and Zupanc 1996). Its lateralmost subdivision, the PPn, consists of a few hundreds of neurons that are of crucial importance for neuronal control of transient modulations of the otherwise extremely constant electric organ discharges (EODs; Heiligenberg et al. 1981; Kawasaki et al. 1988).

Several types of EOD modulations exist. They occur both spontaneously and in the context of social interactions (Larimer and MacDonald 1968; Hopkins 1974; Bullock 1969; Hagedorn and Heiligenberg 1985; Zupanc and Maler 1993; Dunlap et al. 1998; Engler et al. 2000; Engler and Zupanc 2001; Zupanc et al. 2006a; for reviews see Zupanc and Maler 1997; Metzner 1999; Zupanc 2002). One important type of modulation—chirping—is almost exclusively generated by males (Zupanc and Maler 1993; Dulka and Maler 1994). The production of chirps increases greatly in the course of sexual maturation (Hagedorn and Heiligenberg 1985), which, in the natural habitat of South America, is triggered by the onset of the rainy season. In the laboratory, gonadal maturation can be stimulated by imitating the conditions of the tropical rainy season in the aquarium (Kirschbaum 1979).

Experiments employing labeling of mitotic cells with [$^3$H]thymidine or BrdU have shown that the ventricular zone of the CP/PPn is distinguished by high proliferate activity (Zupanc and Zupanc 1992; Zupanc and Horschke 1995; Stroh and Zupanc 1996). On average, roughly 100 cells undergo mitosis within any 2-h period, thus corresponding to approximately 1% of the total cell number of the CP/PPn. Within a few days after mitosis, a good portion of the new cells migrate from the ventricular zone laterally into the adjacent subventricular zone, where at least some of them adopt morphological and immunological characteristics of neurons (Zupanc and Zupanc 1992; Stroh and Zupanc 1996). Experimental evidence suggests that this directed migration of the young cells is mediated by radial glial fibers (Zupanc 2001b).

Remarkably, the areal density of these GFAP-immunopositive radial glial fibers increases with seasonally induced increases in relative gonadal weight in males, but not in females of *Apteronotus* (Gheteu and Zupanc 2002). Despite this increase in radial fiber density and the presumptive larger number of new neurons migrating laterally in males, the total number of cells in the CP/PPn shows a sharp decline with increasing relative gonadal weight in males (but not in females). This unexpected effect is paralleled by a reduction in the volume of the CP/PPn with increasing relative gonadal weight in males but, again, not in females. These seemingly paradoxical effects of sexual maturation in males on radial glial fibers on the one hand, and total number of cells and volume of the CP/PPn on the other, can be explained by such a high turnover rate of the neurons in this complex that the rate of cell death by far exceeds the rate of supply with new neurons. Apoptotic cell death has, indeed, been identified in the CP/PPn (Soutschek and Zupanc 1995). Similarly, degeneration of aging neurons at

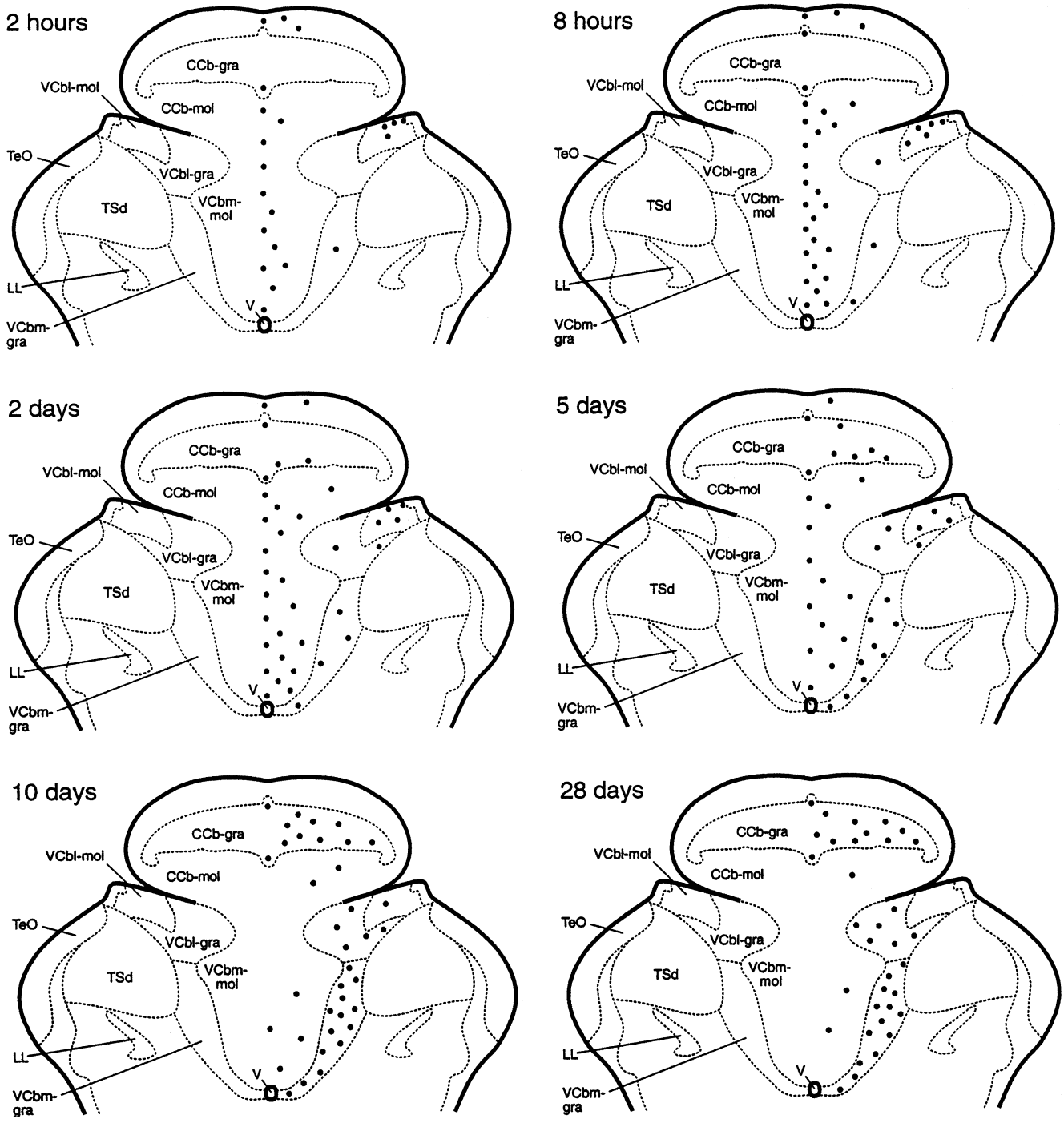



**Fig. 4** Development of cells generated during adulthood in the rostral cerebellum of *A. leptorhynchus*. The distribution of the young cells, identified by labeling with BrdU and indicated by *black dots*, is plotted after post-BrdU administration survival times ranging from 2h to 28 days. The *number of dots* represent approximately the areal density of labeled cells. *CCb-gra* granule cell layer of corpus cerebelli; *CCb-mol* molecular layer of corpus cerebelli; *LL* lateral lemniscus; *TeO* optic tectum; *TSd* dorsal subdivision of torus semicircularis; *V* ventricle; *VCbl-gra* granule cell layer of valvula cerebelli pars lateralis; *VCbl-mol* molecular layer of valvula cerebelli pars lateralis; *VCbm-gra* granule cell layer of valvula cerebelli pars medialis; *VCbm-mol* molecular layer of valvula cerebelli pars medialis (after Zupanc et al. 1996)

times of seasonally induced hyperactivity has been demonstrated in other systems (Polenov and Pavlovic 1978).

The close correlation of the propensity to chirp and the morphology of radial glial fibers in the CP/PPn suggests radial glia to be a critical factor in

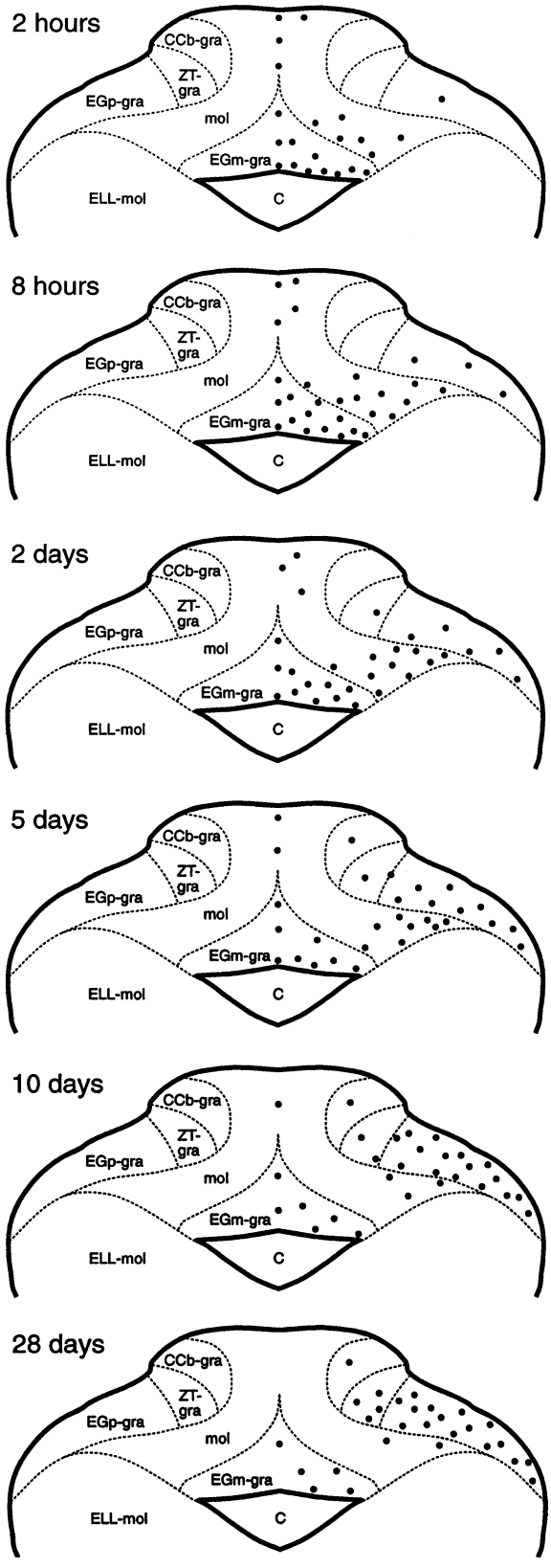



**Fig. 5** Development of cells born during adulthood in the caudal cerebellum of *A. leptorhynchus*. The principle of analysis and representation is identical to the one used in Fig. 4. *C* cerebellomedullary cistern; *CCb-gra* granule cell layer of corpus cerebelli; *EGm-gra* granule cell layer of eminentia granularis pars medialis; *EGp-gra* granule cell layer of eminentia granularis pars posterior; *ELL-mol* molecular layer of electrosensory lateral line lobe; *mol* molecular layer of corpus cerebelli, eminentia granularis pars anterior, eminentia granularis pars medialis, and eminentia granularis pars posterior; *ZT-gra* granule cell layer of transitional zone (after Zupanc et al. 1996)

accommodating seasonally induced changes in chirping behavior. This appears to be mediated through modulation of the final number of neurons guided to their target sites within the brain.

### Cerebellum

In *Apteronotus*, approximately 75% of all new cells produced in the adult brain originate from proliferation zones within the various subdivisions of the cerebellum (Zupanc and Horschke 1995). Qualitative analysis suggests a similar large number of proliferating cells in the cerebellum of other teleosts, including the guppy (Kranz and Richter 1970), gilthead sea bream (Zikopoulos et al. 2000), three-spined stickleback (Ekström et al. 2001), and zebrafish (Zupanc et al. 2005). In *Apteronotus*, in two out of the three major cerebellar subdivisions—the corpus cerebelli and the valvula cerebelli—the vast majority of the cells are generated in the respective molecular layers. In the corpus cerebelli, these proliferation zones are largely restricted to areas at and near the midline in the dorsal and ventral molecular layers. In the valvula cerebelli pars medialis, these zones are found at the midline where they form distinct cell clusters exhibiting high mitotic activity. In the valvula cerebelli pars lateralis, the mitotic cells show a rather wide distribution within the molecular layer. In the third cerebellar subdivision of *Apteronotus*, the eminentia granularis, the new cells are produced in a granule cell layer, the eminentia granularis pars medialis (Zupanc and Horschke 1995; Zupanc et al. 1996). Notably, mitotic cells are also found in the granule cells layer of the lobus caudalis of zebrafish (Zupanc et al. 2005); this brain structure is thought to be homologous to the eminentia granularis pars medialis of gymnotiform fish (Bass 1982). However, the number of labeled cells in the granule cell layer of the lobus caudalis of zebrafish is markedly lower than the number of labeled cells in the eminentia granularis pars medialis in *Apteronotus*.

## Development of the young cells in the cerebellum

The subsequent development of the newly generated cells has been studied in detail in the cerebellum of two teleostean species, *A. leptorhynchus* and the zebrafish. In the following, I will discuss several important aspects of this process.

## Migration

In *Apteronotus*, the new cells migrate from their sites of origin to specific target areas within the three cerebellar subdivisions (Zupanc et al. 1996). These target areas are the granule cell layers in both the corpus cerebelli and the valvula cerebelli partes lateralis and medialis (Fig. 4). In the eminentia granularis, approximately 70% of the cells generated in the pars medialis migrate through the adjacent molecular layer to invade the second granule cell layer, the so-called eminentia granularis pars posterior (Fig. 5). The remaining 30% of the cells continue to reside in the eminentia granularis pars medialis and the adjacent molecular layer. In all three cerebellar subdivisions, the majority of the young cells reach their target areas within 10 days after incorporation of BrdU.

Comparison of these patterns of migration in *Apteronotus* with those found in zebrafish has revealed commonalities, but also interesting differences. Like in *Apteronotus*, in zebrafish the young cells generated in the corpus cerebelli and the valvula cerebelli migrate from their proliferation zones in the molecular layers to the corresponding granular layers (Zupanc et al. 2005). In the caudal cerebellum, however, the migrational pattern differs between the two species. In contrast to *Apteronotus*, there is no evidence in zebrafish that any significant portion of the cells that undergo mitosis in the granular layer of the lobus caudalis—the structure homologous to the eminentia granularis pars medialis of gymnotiform fish—migrate out of this structure. Thus, the new cells produced in the granule cell layer of the lobus caudalis of zebrafish show a similar absence of migratory behavior as do the "resident" 30% of the cells generated in the eminentia granularis pars medialis of *Apteronotus*. On the other hand, approximately 70% of the new cells generated in the eminentia granularis pars medialis of *Apteronotus* that migrate to the granule cell layer of the eminentia granularis pars posterior appear to lack a matching cellular population in zebrafish. This lack might be related to the fact that, in zebrafish, the number of mitotic cells in the granular layer of the lobus caudalis relative to other proliferation zones is significantly smaller than the relative number of S-phase cells in the eminentia granularis pars medialis in *Apteronotus*.

The difference in the pattern of cell proliferation between the two species might be causally linked to the massive electrosensory input received by the granule cell layer of the eminentia granularis pars posterior in gymnotiform fish (Sas and Maler 1987), and the absence of such input in nonelectroreceptive fish. The caudal lobe and its homologous structure in gymnotiform fish may, therefore, be well suited to study, from a comparative point of view, the factors responsible for inter-species differences in proliferation and migration of new cells generated in the adult brain, as well as the functional consequences of these differences.

## Guidance of the migrating young cells

Several lines of evidence, obtained in *Apteronotus*, suggest that, in the course of migration from their proliferation zones to the final target areas, the young cerebellar cells are guided by radial glial fibers (Zupanc and Clint 2003). These fibers form two populations: one immunopositive for glial fibrillary acidic protein (GFAP), the other for vimentin. The morphology and distribution of these two fiber populations are similar but not identical, thus indicating a partial overlap. It is possible that in the course of their development, similar to the situation in mammals (Voigt 1989), the fish radial glia first express vimentin, and then, while the vimentin expression is gradually reduced, an increasing amount of GFAP will be produced. However, final verification of this hypothesis awaits further experimentation.

In all three cerebellar subdivisions, both the vimentin-positive fibers and the GFAP-expressing radial glial fibers delineate the path taken by the young cells in the course of their migration. For instance, in the corpus cerebelli such fibers originate from the tips formed at the midline by protrusion of granule cells from the granule cell layer into the dorsal and ventral molecular layers. Initially, the fibers run along the midline, but shortly before reaching the pial surface in the dorsal molecular layer, they make lateral turns and continue to run in lateral directions for up to several hundreds of micrometers (Fig. 6). This fiber course exactly matches the route through which cells born in the molecular layer migrate into the granule cell layer. A similar matching between the distribution and orientation of GFAP- and vimentin-positive radial glial fibers on the one hand, and the migrational path taken by young cells on the other, is observed in the other two cerebellar subdivisions.

The hypothesis that radial glial fibers in the cerebellum provide scaffolding for the migrating young cells receives further support by the results of double-labeling experiments. Intraperitoneal injection of BrdU, followed by sacrifice of the fish 2 days postadministration (i.e., at a time when the young cells exhibit maximum migratory activity) reveals elongated BrdU-labeled cells in close apposition to GFAP-labeled radial glial fibers (Gheteu and Zupanc 2001; Fig. 7).

## Regulation of the number of new cells by apoptotic cell death

After arrival at their target sites within the cerebellum of *Apteronotus*, the areal density of BrdU-labeled cells drops by roughly 50% in the period 4–7 weeks after their generation (Zupanc et al. 1996; Ott et al. 1997). This decrease is thought to be due to apoptotic cell death. Such a mechanism of cell elimination has been suggested by experiments in which terminal deoxynucleotidyl-transferase-mediated dUTP-biotin nick end-labeling (TUNEL) of 3′-OH ends of DNA was employed (Soutschek and Zupanc 1996). The latter

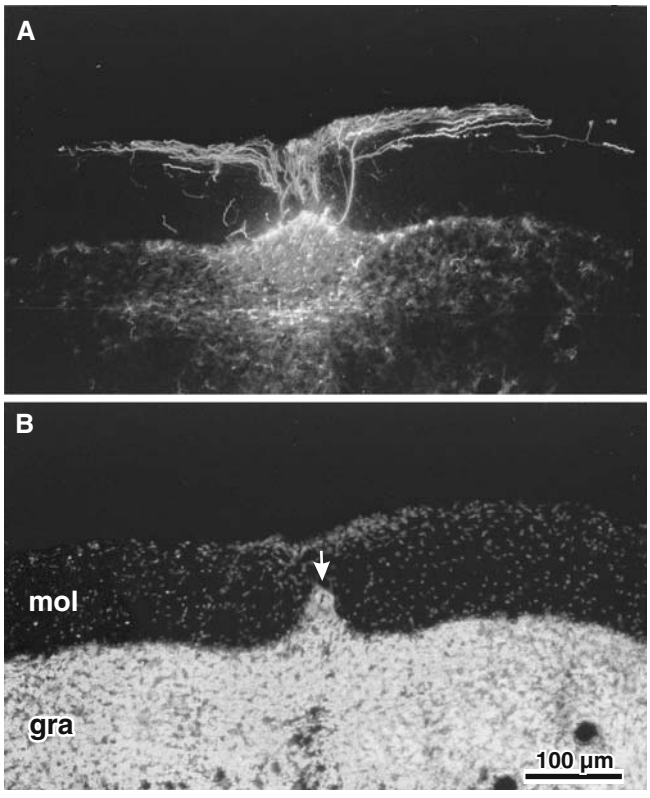



**Fig. 6** Radial glial fibers, identifed by immunostaining against GFAP, in the dorsal portion of the corpus cerebelli, in *A. leptorhynchus* (**a**). At the midline of the dorsal granule (*gra*) cell layer, the granule cell mass protrudes into the dorsal molecular (*mol*) layer by forming a tip (*arrow*), as evident in the counterstain produced by applying the nuclear dye DAPI to the section (**b**). This tip is the origin of long fibers that initially take a trajectory parallel to the midline. As soon as they get close to the dorsal surface of the brain, these fibers split into two populations turning laterally to the left and right side, respectively. Following this turn, they continue to run parallel to the dorsal surface of the brain over a few hundred micrometers. As BrdU-labeling studies have shown, this fiber course exactly matches the route alongside which cells born in the molecular layer migrate into the granule cell layer (from Zupanc 2001a)

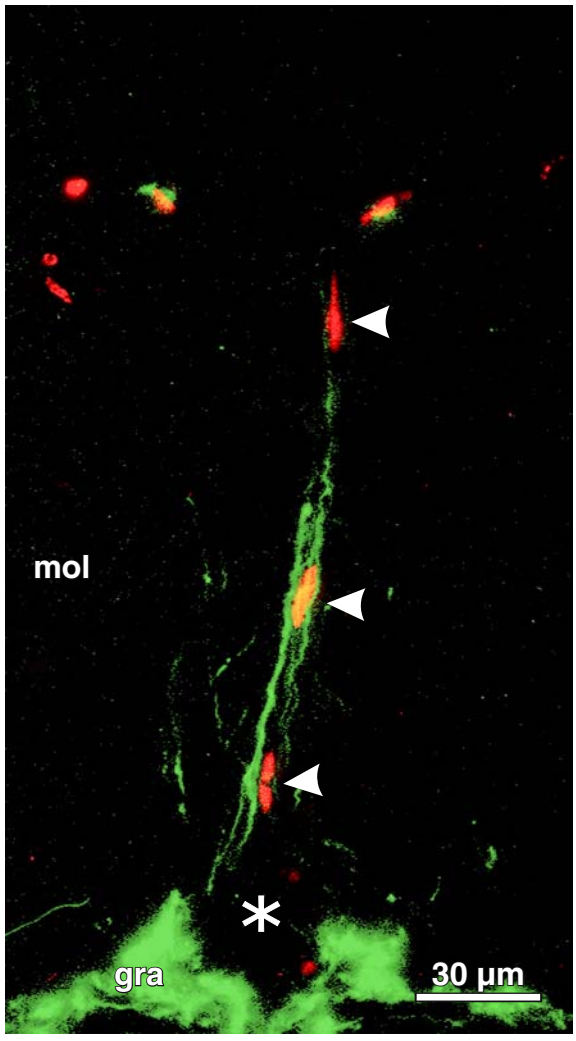



**Fig. 7** Guidance of young cells by radial glial fibers in the cerebellum of the *A. leptorhynchus*. Two days after the administration of the S-phase marker BrdU, the fish was killed, and the transverse section through the dorsal portion of the corpus cerebelli was immunostained against both BrdU (*red color*) and GFAP (*green color*). Long GFAP-positive radial glial fascicles run at the midline of the mol layer through the so-called dorsal tip (*asterisk*) into the gra cell layer. Several BrdU-labeled cells, indicated by *arrowheads*, are closely apposed to the radial glial fibers (from Zupanc 2001a)

approach is based on the identification of DNA fragmentation as a feature characteristic of apoptosis (Modak and Bollum 1972; Gavrieli et al. 1992; Surh and Sprent 1994).

Studies applying TUNEL to brain sections of *Apteronotus* have shown that a large number of cells continuously undergo apoptosis in the granular layers of the three subdivisions of the cerebellum (Soutschek and Zupanc 1996). By contrast, the number of apoptotic cells is low in the molecular layers. This suggests that apoptosis is used as a mechanism to regulate the number of young cells after they have reached their target areas.

A similar mechanism has been demonstrated in other brain systems, particularly those of mammals, during embryonic development. In these systems, apoptosis leads to the elimination of young cells which, after arrival at the target site, have failed to make proper connections with other neurons and to receive adequate amounts of specific survival factors produced by cells in the target area (Raff 1992; Raff et al. 1993).

## Long-term survival of new cells

In *A. leptorhynchus*, it is likely that most of the cells that have survived the massive wave of apoptotic cell death 4–7 weeks after their generation continue to exist for the rest of the fish's life. This is suggested by experiments in which post-BrdU administration survival times of up to 440 days were employed (Zupanc et al. 1996; Ott et al. 1997). The latter period is equivalent to roughly half the adult life span of this species in captivity.

Long-term survival of new cells of at least 292 days has been demonstrated in a large number of brain regions of the zebrafish (Zupanc et al. 2005). The entire life span of outbred zebrafish is roughly 3.5 years (Gerhard et al. 2002), so that a survival time of 292 days covers approximately one-fourth of the entire life span and one-third of the remaining life expectancy after administration of BrdU (the fish used in the experiments received a single pulse of this thymidine analogue at an approximate age of 9–12 months).

A similar long-term persistence of new cells has been observed in the mammalian brain as found in *Apteronotus* and zebrafish. In the granular layer of the hippocampus of adult mice, new neurons survive for at least 11 months (Kempermann et al. 2003), which is equivalent to roughly half the life span of mice.

In *Apteronotus*, the long-term survival, together with the continuous production of new cells, leads to a permanent growth of the entire brain, except for very old fish in which the growth rate appears to reach a plateau. This parallels the continuous growth of the body in this species. A quantitative analysis has demonstrated that, while the body weight of the fish increases from 1 to 16 g, the total number of brain cells doubles from $5\times10^7$ to $1\times10^8$ (Zupanc and Horschke 1995).

### Neuronal differentiation

A central question arising in the context of the generation of new cells in the adult fish brain concerns their cellular identity. This issue has been addressed in detail with regard to the zebrafish (Zupanc et al. 2005). The approaches used include combination of anti-BrdU immunohistochemistry with immunostaining against the neuron-specific protein Hu. Such double-labeling experiments have revealed that, at post-BrdU administration survival times of 9 months, approximately 50% of the BrdU-labeled cells in the zebrafish brain express this neuron-specific protein. Such neurons are particularly abundant in the dorsal telencephalon, including the region presumably homologous to the mammalian hippocampus (see ''Cell proliferation—Dorsal telencephalon'').

Since after post-BrdU administration survival times of 7–10 months approximately 50% of all BrdU-labeled cells are situated in the granular layers of the corpus cerebelli and the valvula cerebelli partes lateralis and medialis, it is reasonable to assume that at least a part of the new cerebellar cells develop into granule cell neurons. Unfortunately, any of the anti-Hu antibodies tested thus far has failed to immunostain cerebellar granule cells. Therefore, using an alternative approach, the possible differentiation of young cerebellar cells into granule cell neurons has been examined by combination of BrdU labeling and neuronal tract tracing. Such experiments, conducted in both *Apteronotus* (Zupanc et al. 1996) and zebrafish (Zupanc et al. 2005), have indeed provided evidence in favor of the hypothesis that at least some of the cells generated in the adult cerebellum develop into granule cell neurons. Just as in these studies the BrdU-labeled granule cells were retrogradely traced by application of tracer substance to the molecular layers, the experiments also indicate that these cells have developed axons traveling from their somata in the granule cell layers to the corresponding molecular layers. Thus, it appears that they have integrated into the existing neural network of cerebellar neurons. It is unknown whether the axons of the newly generated granule cells make proper synaptic connections with other cells, such as Purkinje cells. It also remains to be examined whether the new granule cells are functional, and whether their physiological properties are similar to those of the ''older'' granular neurons.

## Towards a functional understanding of adult neurogenesis: numerical matching of central and peripheral elements

It has been proposed that, besides involvement in the accommodation of behavioral plasticity (see ''Cell proliferation—Central posterior/prepacemaker nucleus'', above) and in the replacement of damaged neurons during neuronal regeneration (see ''Neuronal regeneration—Recruitment of new cells'', below), adult neurogenesis in fish plays an important role in the numerical matching of neurons in the central nervous system and of sensory and motor elements (Zupanc 1999a, 2001a). The latter hypothesis is based on the observation that many fish species grow continuously throughout life. Although mammals also grow during postembryonic stages of development, there is a distinct difference between these two taxonomic classes. Whereas in mammals growth is the result of an increase in size but not in number of individual muscle fibers, in fish it is the number of muscle fibers that increases (Koumas and Aksten 1995). It is, therefore, possible that the increase in the number of peripheral motor elements prompts a concomitant increase in the number of central neurons involved in neural control of associated muscle activity.

Along similar lines, the number of sensory receptor cells, receptor organs, or receptor units in the periphery has been shown to increase with age in several species of fish. Such a formation of new sensory elements has been demonstrated for sensory hair cells in the inner ear of sharks (Corwin 1981), for retinal cells in the eyes of goldfish (Johns and Easter 1977), and for electrosensory receptor organs in the gymnotiform fish *Sternopygus darienses* (Zakon 1984). By contrast, the production of sensory cells in mammals appears to cease by the end of gestation (e.g., Ruben 1967), except for primary sensory neurons of the olfactory epithelium (Graziadei and Graziadei 1979). Thus, as it occurs in the formation of new motor elements, the continuous increase in the number of sensory elements in fish may lead to the generation of new neurons involved in the processing of sensory information, thus ensuring a numerical matching of central neurons and peripheral sensory elements.

The numerical matching hypothesis could explain the enormous mitotic activity in the fish cerebellum. Based on anatomical, physiological, and behavioral experiments, the cerebellum, notably of weakly electric fish, has been proposed to play an important role not only in the well-established function of control and coordination of movements, but also in sensory processing, particularly by tracking movements of objects around the animal, and by the generation and subtraction of sensory expectations (for reviews see Paulin 1993; Bell et al. 1997). On the other hand, the lack of adult neurogenesis in the mammalian cerebellum could be causally linked to the absence of changes in the number of muscle fibers and sensory elements during periods of growth in the periphery.

At the central level, numerical matching has been demonstrated between the presynaptic granule cell population and its postsynaptic target neurons, the Purkinje cells, by making use of the neurologically mutant mouse strain lurcher. The cerebella of lurcher heterozygotes are characterized by early postnatal degeneration of several cell types, beginning with the loss of Purkinje cells during the second week of life. This stage is followed by degeneration of granule and olivary neurons and Bergmann glia (Swisher and Wilson 1977; Caddy and Biscoe 1976). In wild-type mice, there is a constant ratio of about 175 granule cells for each Purkinje cell. In Lurcher chimeric mice, variation in the number of Purkinje cells is genetically caused, whereas the loss of granule cells appears to be a secondary, phenotypic consequence due to Purkinje cell loss (Wetts and Herrup 1983).

In fish, there is some experimental evidence supporting the hypothesis that changes in the number of peripheral sensory elements lead to changes in the production of the corresponding central elements. Raymond et al. (1983) found in goldfish that permanent removal of the optic input by enucleation of the eye results in sustained depression of mitotic activity in the tectal proliferation zone on the denervated side compared to the intact one. Temporary denervation by optic nerve crush initially has a similar effect, but upon reinnervation of the tectum by the regenerating optic fibers proliferation is enhanced on the experimental side compared to the control side.

## Neuronal regeneration

The enormous potential of teleost fish to continuously produce new neurons in the intact brain during adulthood is closely related to a second phenomenon by which fish are distinguished from mammals: the ability to replace neurons lost to injury by newly generated ones. This so-called neuronal regeneration has been studied in *A. leptorhynchus* more than in any other teleostean species (for reviews see Zupanc 1999a, 2001a; Zupanc and Clint 2003).

### The lesion paradigm

Investigations of brain repair in *Apteronotus* have been greatly facilitated by the availability of a well-established lesion paradigm. This paradigm takes advantage of the prominent position of the corpus cerebelli, which forms a roof on top of the brain, except over the telencephalon. Cerebellar lesions can, therefore, be made by puncturing the skull of the anesthetized fish with a sterile surgical blade, without damaging other parts of the brain (Fig. 8). Guided by landmarks on the fish's head, lesions are applied in a defined way, resulting in an approximately 1-mm deep cut traveling in parasagital direction and encompassing both the dorsal molecular layer and the granular layer of the corpus cerebelli.

Nissl stains of sections taken through the corpus cerebelli after various survival times have shown that the initial clearly visible path caused by the stab wound gradually becomes reduced with longer post-lesioning survival times, until it disappears after a couple of weeks (Zupanc et al. 1998). This indicates rapid and efficient neuronal wound healing.

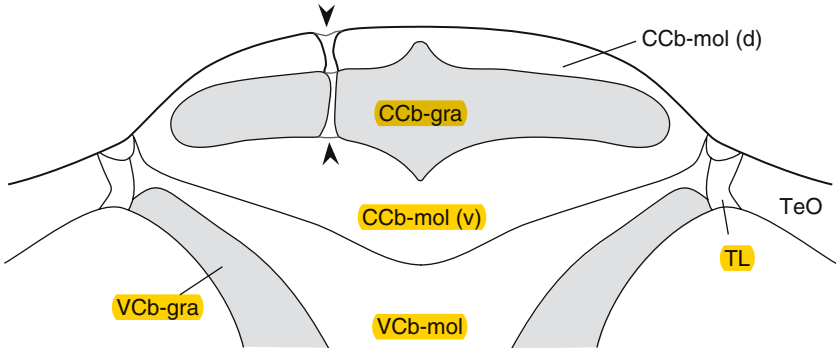



**Fig. 8** Schematic representation of the lesioning paradigm. The stab-wound lesion (*arrowheads*) is applied unilaterally to the corpus cerebelli such that (i) the scalpel penetrates both the dorsal molecular layer [CCb-mol(d)] and the granular layer (CCb-gra) and (ii) the path of lesion is located roughly halfway between the midline of the brain and the lateral edge of the granule cell layer. *CCb-mol(v)* ventral molecular layer of corpus cerebelli; *TeO* optic tectum; *TL* torus longitudinalis; *CCb-gra* granule cell layer of valvula cerebelli; *VCb-mol* molecular layer of valvula cerebelli (from Zupanc et al. 2006b)

## Elimination and removal of damaged cells

Detailed analysis has shown that the tissue repair is accomplished by two major processes. The first process involves the rapid elimination of damaged cells through apoptosis (Zupanc et al. 1998), followed by removal of the resulting cellular debris by the action of microglia/macrophages (Zupanc et al. 2003). Apoptosis was originally shown to regulate cell numbers in the mammalian brain during embryonic development (Raff et al. 1993). It is believed that this type of cell death exerts a similar function in the intact fish brain during postnatal development (Soutschek and Zupanc 1995, 1996; see "Development of the young cells in the cerebellum—Regulation of the number of new cells by apoptotic cell death", above). The involvement of apoptosis in the process of elimination of injured cells is remarkable because this contrasts with the situation in mammals. In the latter taxon, besides apoptosis (for reviews see Beattie et al. 2000; Vajda 2002), necrosis occurs and appears to be the predominant type of cell death following lesions (Kerr et al. 1987). Necrosis in mammals commonly leads to inflammation at the site of the injury (for review see Kerr et al. 1995). This inflammatory response triggers further necrotic events, thus gradually transforming the site of injury into large cavities devoid of cells (Zhang et al. 1997). These cavities are typically bordered by scars that act as mechanical and biochemical barriers preventing the ingrowth of nerve fibers and the migration of cells into the lesion site (for review see Reier et al. 1983).

In *Apteronotus*, the first apoptotic cells are detectable as early as 5 min after application of a lesion to the corpus cerebelli (Zupanc et al. 1998). Thirty minutes after the lesion, the number of cells undergoing apoptosis reaches maximum levels. At 2 days post-lesioning survival time, the number of apoptotic events, as indicated by TUNEL, starts to gradually decline until background levels are reached at approximately 20 days after the lesion (Fig. 9).

Apoptosis is characterized by cell shrinkage, nuclear condensation, and production of membrane-enclosed

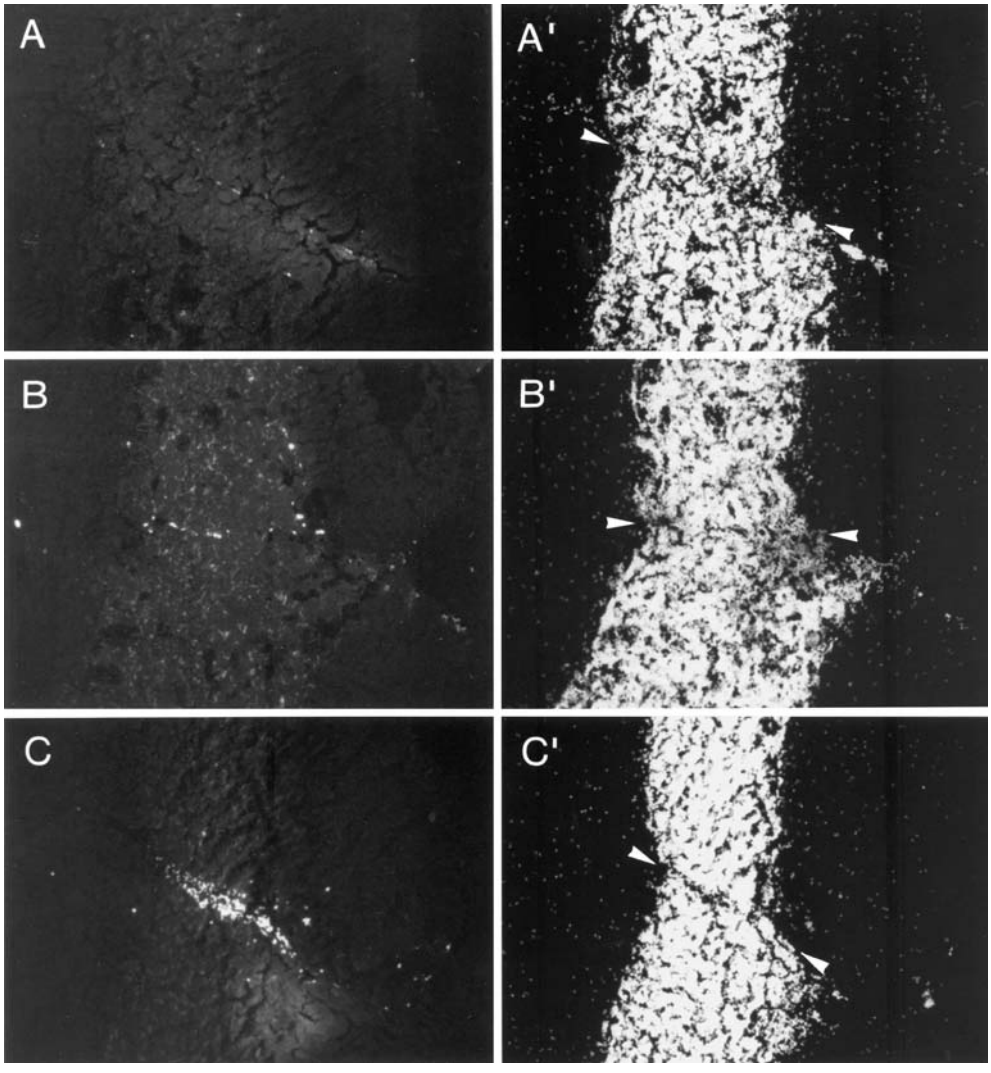
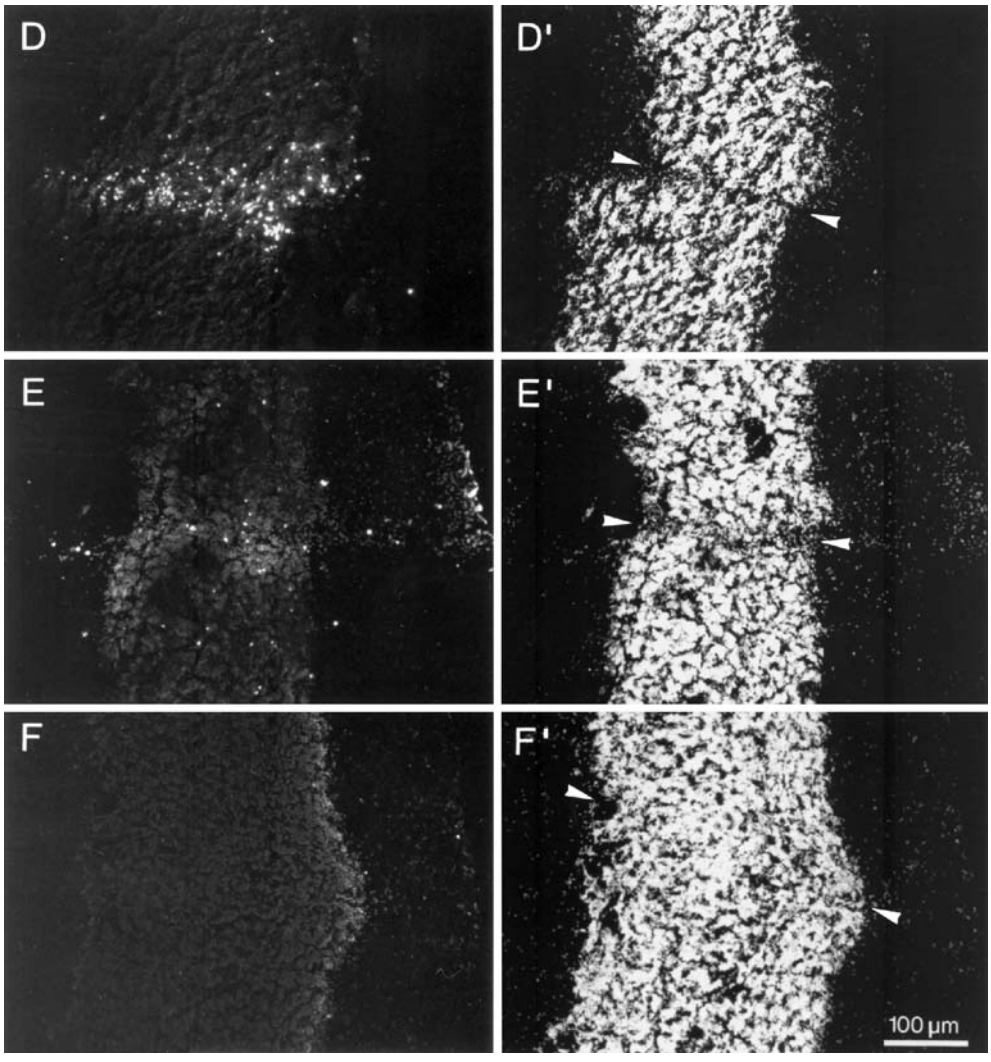



**Fig. 9** TUNEL-positive cells at the site of the lesion in the corpus cerebelli after survival times of 5 min (**a**), 15 min (**b**), 30 min (**c**), 1 day (**d**), 5 days (**e**), and 20 days (**f**). The location of the lesion is indicated by *arrowheads* in the DAPI counterstaining (**a′**–**f′**). The number of TUNEL-positive cells dramatically increases within the first 30 min, stays at high levels for another day, and then gradually declines to background levels (from Zupanc et al. 1998)

**Fig. 9** (Contd.)




particles that are digested by other cells. Most significantly, the side-effects that accompany necrosis, such as inflamation of the surrounding tissue, are typically absent in apoptosis. This suggests that the use of this "clean" type of cell death for the elimination of damaged cells is an essential component of the enormous regenerative capability of *Apteronotus*.

Approximately 3 days after application of the lesion, the areal density of microglia/macrophages, identified by tomato lectin binding, starts to increase at and near the lesion site (Zupanc et al. 2003). A similar increase, although less pronounced, is also seen in the contralateral hemisphere. The areal density of microglia/macrophages reaches maximum levels at approximately 10 days post lesion and returns to background levels approximately 1 month after the lesion. The emergence of microglia/macrophages shortly after the number of apoptotic cells has reached maximum levels supports the notion that the former cells are involved in the removal of cellular debris caused by apoptotic cell death.

## Generation of new cells

The second major process mediating brain repair in *Apteronotus* encompasses replacement of the neurons that are eliminated through apoptosis with newly generated ones. These neurons are recruited from two sources (Zupanc and Ott 1999). First, new cells are produced in response to the injury in proliferation zones near the lesion; these areas of mitotic activity are detectable only after injury. Second, new cells are recruited from the pool of continuously generated undifferentiated cells.

The vast majority of cells of either of the two populations are produced 1–10 days following the lesion, with the maximum proliferative activity occurring at 5 days post injury. This upregulation of proliferative activity is thought to be due to an inductive effect exerted by the injury. In addition, cells that are born before the lesion also contribute to the restoration of damaged tissue. BrdU-labeling experiments have shown that cells generated as early as 2 days prior to lesioning

of the cerebellum still participate in the process of regeneration (Zupanc and Ott 1999).

The observation that cells generated before the occurrence of the injury can replace damaged neurons suggests a direct relationship between the continuous cell proliferation in the intact brain and the process of neuronal regeneration. The continuous provision of a pool of undifferentiated cells in the intact brain appears to enable fish to recruit new cells much more rapidly in the event of injury than would be possible by recruiting only cells that are generated in response to injuries.

## Migration and differentiation of new cells

As an important step in the further process of recruitment of the new cells for repair of the lesioned tissue, these cells have to migrate from the site of their production to the site of injury. Several lines of evidence suggest that this is achieved through guidance by radial glial fibers. Approximately 8 days following the lesion, the density of radial glial fibers, identified by morphological criteria and immunohistochemical staining against GFAP, increases greatly compared to the levels found in the intact cerebellum (Clint and Zupanc 2001). This effect is restricted to the site ipsilateral to the lesion, and occurs at particularly high levels at and near the site of injury. The increase in radial fiber density is followed by a rise in the density of young cells, in the same cerebellar areas, approximately 2 days later. This arrival of young cells *after* the appearance of radial glial fibers, as well as the frequently observed close apposition of young cells to radial glial fibers, support the notion that radial glial fibers provide a scaffold to guide the young cells in the course of their migration from the site of origin to the site of injury.

Like the increase in areal density of GFAP-expressing radial glial fibers, the expression of vimentin in fibers at the site of the lesion and in the remaining ipsilateral molecular layer is upregulated in a specific spatio-temporal fashion (Clint and Zupanc 2002). The areal density of vimentin-positive fibers increases significantly 15 days after application of a mechanical lesion to the corpus cerebelli and remains elevated throughout the time period of up to 100 days examined. It is possible that the vimentin-expressing fibers also represent a sub-population of radial glia-like cells. Although there appears to be a certain degree of overlap between the two fiber populations, GFAP-positive fibers are typically found at greater distances from the lesion site in the ipsilateral hemisphere and exhibit a more elongated morphology than vimentin-positive fibers. Since the majority of the new cells arrive at the lesion site *before* the upregulation of vimentin, it is unlikely that this intermediate filament protein is involved in guidance of the migrating young neurons. Instead, vimentin may play a role in later developmental functions, such as promotion of cellular survival, differentiation, and/or outgrowth of dendrites.

Combination of BrdU labeling with neuronal tract tracing has shown that at least some of the new cells that replace damaged neurons are cerebellar granule cells (Zupanc and Ott 1999). Because in this study the granule cell neurons have been identified by deposition of the tracer substance into the molecular layer of the corpus cerebelli and through subsequent retrograde transport of the tracer to somatic regions, this result also shows that these cells have established axonal projections with the associated molecular layer.

## Towards a molecular understanding of brain regeneration: the single-protein approach

One of the central goals of research on neuronal regeneration in fish is to gain an understanding of the molecular mechanisms mediating this phenomenon. The traditional approach used to achieve this goal has been to select a single candidate protein and examine possible changes in its abundance after injury. The selection of this protein is, in most instances, based on one or several of the following three analogous conclusions: First, a protein involved in neuronal regeneration of one species might also be involved in neuronal regeneration of other species. Second, a protein that plays a functional role in wound healing of one organ (e.g., skin or liver) might also participate in the regeneration of other types of tissue, including neuronal ones. Third, a protein that directs certain steps of embryonic or adult development in the intact organism might also exert a function in the process of regeneration.

Based on these conclusions, several regeneration-associated factors have been identified in the corpus cerebelli of *Apteronotus*. Among them, the best-examined one is the neuropeptide somatostatin. Somatostatin-like immunoreactivity (Sas and Maler 1991; Zupanc et al. 1991a; Stroh and Zupanc 1993, 1995, 1996) and somatostatin messenger RNA (Zupanc et al. 1991b) are widely distributed in the brain of *Apteronotus* and the closely related genus *Eigenmannia*. The endogenous ligand interacts with specific receptors, as indicated by the demonstration of somatostatin receptor-binding sites (Zupanc et al. 1994) and the molecular cloning and pharmacological characterization of a somatostatin receptor resembling the mammalian $sst_3$ receptor subtype (Zupanc et al. 1999; Siehler et al. 1999, 2005).

In the intact corpus cerebelli, very few cells displaying somatostatin-like immunoreactivity are found. However, their numbers increase dramatically at the lesion site 1 day after application of the stab wound (Zupanc 1999b). The number of cells displaying somatostatin-like immunoreactivity in this area reaches peak levels at 2 days, followed by a rapid decline to background levels between 5 and 10 days post lesion.

Since the time period during which somatostatin is upregulated coincides with the period during which most of the cells later incorporated at the site of the lesion are generated, it is possible that this neuropeptide is involved in the regulation of cell proliferation. Indeed, a growth-inhibiting action of somatostatin is well established for numerous tumors (for review see Pollak and Schally 1998), and for the regeneration of several peripheral organs (Kokudo et al. 1992; Thompson et al. 1993; Zieleniewski and Zieleniewski 1993; Bufalari et al. 1996). Somatostatin appears to exert such a function though a cell cycle block due to $G_1$ arrest (Mascardo and Sherline 1982), or through induction of apoptosis (Szende et al. 1989; Srikant 1995).

Alternatively, somatostatin may act as a trophic factor-like substance by influencing the early stages of differentiation of the young granule cell neurons. Evidence for such a function stems from developmental studies in mammals. In the cerebellum, somatostatin-binding sites are transiently expressed during a restricted period shortly after birth when the cerebellar granule cells are formed. Medium supplemented with somatostatin induces neurofilament synthesis in cultured granule cells (Taniwaki and Schwartz 1995; Schwartz et al. 1996), thus supporting the notion that this neuropeptide could acts as a differentiation factor.

## Towards a molecular understanding of brain regeneration: large-scale identification of regeneration-associated proteins

Until recently, only the single-protein approach was available to identify regeneration-associated proteins in the fish brain. This approach requires the availability or the generation of specific antibodies to examine protein abundance by immunohistochemistry in the fish brain. The identification of such antibodies can be a cumbersome process, as most commercially available antibodies are directed against mammalian orthologues, and might thus fail to show specific labeling in fish tissue. Possible changes in protein abundance can be found by examining sections taken from the site of injury at various post-lesion survival times. Typically, the initial screening involves the examination of tissue collected at 5–10 different survival times, thus resulting in a time-consuming and labor-intensive procedure. As a consequence, it is almost impossible to identify a sufficiently large number of proteins to adequately reflect the complexity of the molecular processes that direct the restoration of the tissue after injury. Moreover, as this approach is based on analogous conclusions, it is not suitable to identify novel proteins.

To overcome these difficulties, an alternative approach was employed that enables the investigator to conduct a large-scale identification of novel protein candidates involved in the repair of the teleostean brain (Zupanc et al. 2006b). This approach is based on a combination of the cerebellar lesion paradigm with differential proteome analysis. For our survey, we chose a post-lesion survival time of 3 days because a previous study had demonstrated the importance of this time point, especially for the recruitment of newly generated cells replacing the ones lost to injury (Zupanc and Ott 1999). At 3 days following a lesion, cell proliferation is dramatically upregulated, both in areas near the lesion and in proliferation zones that continuously generate new cells in intact fish.

For the proteome analysis, abundance of proteins in tissue from the site of the lesion was compared with the abundance of the same proteins in an equivalent region of the intact cerebellum. Two-dimensional gel electrophoresis of protein extracts from these two types of tissue samples revealed nearly 800 protein spots (Fig. 10). Out of this total number of spots, spot intensity was significantly increased by at least twofold in 30 spots and decreased to at least half the intensity of intact tissue in 23 spots (Fig. 11). Since this investigation was restricted to the examination of cytosolic proteins, as well as proteins with isoelectric points between 4 and 7, the result indicates that the total number of proteins potentially involved in regeneration

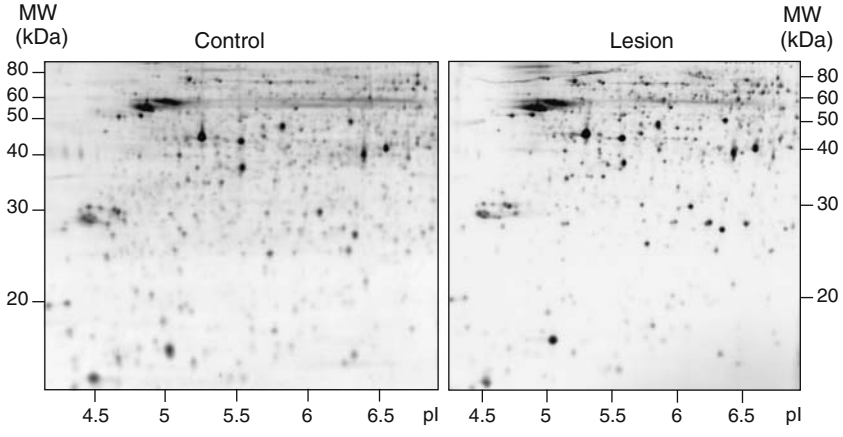



**Fig. 10** Images of two-dimensional gels of proteins from the intact corpus cerebelli ("Control") and lesioned corpus cerebelli ("Lesion") at a post-lesion survival time of 3 days in *A. leptorhynchus* (from Zupanc et al. 2006b)

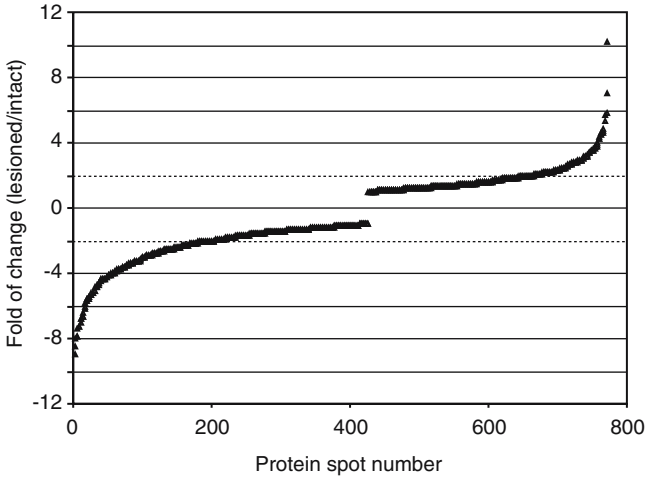



**Fig. 11** Scatter plot of the fold changes of 772 polypeptide spots in the corpus cerebelli of *A. leptorhynchus* 3 days after a lesion compared to unlesioned controls. Decrease in protein abundance is indicated by negative changes and increase by positive changes. Proteins are sorted in ascending order of the spot intensity change. The analysis is based on a comparison of the averaged spot intensities of four 2D gels from lesioned brains and three 2D gels from intact brains. The *dotted lines* indicate the +2-fold and −2-fold threshold (from Zupanc et al. 2006b)

of the injured cerebellum at the post-lesion survival time examined may well exceed 100. It will be an enormous challenge to identify these proteins and to explore their functions!

The proteins associated with 24 of the 53 spots that showed significant intensity differences after application of a lesion to the cerebellum in *Apteronotus* could be identified by peptide mass fingerprinting and mass spectrometry/mass spectrometry fragmentation. Table 1 lists these identified protein spots, together with the changes found in the lesioned corpus cerebelli, relative to an equivalent area in the intact corpus. Also included in this list are the proposed functions of these proteins in the process of tissue regeneration. Detailed analysis has shown that these proteins can be divided into three major groups: The first group includes cytoskeletal proteins essential for the formation of new cells and proteins mediating the correct assembly of these structural proteins. The second group is comprised of proteins putatively involved in cell proliferation, cellular motility, neuroprotection, and energy metabolism. The third group consists of a single protein, bone marrow zink finger 2. This protein is of particular interest because it regulates the transcription of genes of other

**Table 1** Differentially expressed proteins 3 days after application of a lesion to the corpus cerebelli of *Apteronotus leptorhynchus*. The proteins were identified by proteome analysis (data from Zupanc et al. 2006b)

| Protein name | Fold change | $M_r$/pI | Proposed function |
|---|---|---|---|
| $\beta$-Actin | 2.1 | 42/5.3 | Cytoskel etal protein of axons |
| $\beta$-Tubulin | 2.9 | 50/4.8 | Cytoskeletal protein of axons and endothelial cells |
| $\beta$1-Tubulin | 3.6 | 50/4.8 | Cytoskeletal protein related to neurogenesis |
| Keratin-10 | 0.5 | 59/5.1 | Intermediate filament protein; negative regulation of cell proliferation |
| Chaperonin containing tailless-complex polypeptide 1, subunit $\varepsilon$ | 2.4 | 60/5.4 | Chaperoning of cytoskeletal proteins |
| Tropomodulin-3 and -4 | 2.3 | 40/4.7 | Capping of slow-growing ends of actin filaments |
| Bullous pemphigoid antigen 1 | 4.2 | 38/6.4 | Linking of actin with intermediate filament proteins |
| Myosin heavy chain | 2.5 | 22/5.5 | Cellular motility of axons |
| B2-Lamin | 2.9 | 68/5.4 | Nuclear assembly during mitosis |
| 78000 dalton glucose-regulated protein | 3.7 | 73/5.0 | Neuroprotection by reducing apoptotic cell death |
| Glutamine synthetase | 2.2 | 42/6.4 | Neuroprotection by converting glutamate into glutamine |
| Cytosolic aspartate aminotransferase | 0.5 | 47/6.7 | Neuroprotection by reduction of glutamate and aspartate |
| $\alpha$-Enolase | 2 | 48/6.2 | Energy metabolism |
| $\beta$-Enolase | 2.1 | 47/7.1 | Energy metabolism |
| F-ATP synthase _-subunit | 2.1 | 55/5.1 | Energy metabolism |
| Vacuolar adenosine triphosphatase | 2.5 | 69/5.4 | Possibly regulation of cellular growth |
| Calcineurin | 0.4 | 60/5.6 | Regulation of apoptotic cell death |
| 70-kDa heat-shock cognate protein | 2.8 | 71/5.3 | Protein folding; possibly regulation of development |
| Phosphoglycerate kinase | 2.1 | 43/5.9 | Glycolysis; modulation of DNA polymerization |
| Creatine kinase | 2.3 | 43/5.5 | Energy metabolism; regulation of cell proliferation |
| Bone marrow zinc finger 2 | 0.4 | 85/9.7 | Transcriptional regulation |
| Regeneration-associated protein 1 (similarities to steroid sensitive gene-1 protein from *Danio rerio*) | 4.2 | 100/9.7 | ? |
| Regeneration-associated protein 2 (similarities to protein CG9699-PG from *Drosophila melanogaster*) | 0.4 | 50/6.2 | ? |

$M_r$, molecular weight (kDa); pI, isoelectric point

proteins. As such, it is a promising candidate for a long-sought factor—the master regulator in the repair process. Such a master protein could have great potential for biomedical applications in the treatment of neurodegenerative diseases and injuries of the central nervous system.

## A biomedical perspective

The study of neurogenesis and neuronal regeneration in the adult fish brain bears enormous potential for future research, not only to arrive at a better understanding of the biological function(s) and the evolutionary constraints of the continued generation of new neurons in the adult vertebrate brain in general, but also to catalyze important advances in biomedicine. As shown earlier, the limits of the mammalian brain to replace neurons lost to injury or neurodegenerative disease by newly generated ones contrast with the enormous capability of the adult fish brain for neuronal regeneration. This failure of mammals to effect efficient brain repair appears to be caused by the absence of proper permissive signals stimulating generation of new cells and regulating their development, rather than the lack of an intrinsic potential for neurogenesis or the presence of an inhibitory signal.

Support for this hypothesis comes from the observation that the potential to produce new neurons is present in many more areas of the adult mammalian brain than just the hippocampus and olfactory bulb. Multipotent stem cells, although quiescent in vivo, have been isolated from various areas of the brain and spinal cord. Treatment of cultures of these cells with a combination of epidermal growth factor and basic fibroblast growth factor induces proliferation, self-renewal, and expansion of such cells (Weiss et al. 1996). Thus, one major mechanism that suppresses adult neurogenesis in mammals appears to be the absence of permissive signals for proliferation and further development of the intrinsic stem cells.

By comparison, such regeneration-permissive factors are evidently present in the adult fish brain. Since the mechanisms controlling brain development are very similar among vertebrates, identification of the molecular factors that mediate neuronal regeneration in fish is likely to provide important insights into the factors limiting this phenomenon in mammals. Such a comparative approach could, therefore, be used to define new therapeutic strategies to finally overcome the limits of the mammalian central nervous system.

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