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Cell proliferation in the olfactory bulb of adult freshwater teleosts*

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INTRODUCTION

Autoradiographic techniques using tritiated thymidine provide information on the time of origin of new cells and position of their precursors. [^{3}H]thymidine is almost exclusively incorporated into the nuclei of premitotic cells (Angevine, 1965) giving an insight of the cell population that, at the time of the injection, is synthesising new DNA.

Autoradiographic studies on the neurogenesis of the olfactory bulb have been carried out mainly in mammals (Altman, 1967, 1969; Bayer, 1983, 1986; Hinds, 1968*a*, *b*; Kaplan & Hinds, 1977). In adult rats, Altman (1962) proposed that neurogenesis takes place in the cerebral cortex. Later, Altman (1963, 1969) and Altman & Das (1965) demonstrated, using histological and autoradiographic techniques, significant neurogenesis in the granule cell populations of both hippocampus and olfactory bulb. In addition, Kaplan & Hinds (1977) studied light autoradiographs electron microscopically in adult rats and showed that these newly formed cells have the ultrastructural characteristics of neurons.

The data relating to cell proliferation in teleosts refer mainly to the optic tectum during postembryonic growth (Raymond & Easter, 1983; Raymond, Easter, Burnham & Powers, 1983), or in adult animals, under experimental conditions (Johns, Easter, Burnham & Powers, 1977; Kirsche & Kirsche, 1961; Stevenson, 1976, 1977; Stevenson & Yoon, 1978). However, no data have been presented concerning cell proliferation in the olfactory bulb of teleosts.

The data reported for the olfactory bulb of mammals cannot be applicable to the teleost olfactory bulb. Thus, in the olfactory bulb of vertebrates, there is a common transmitter and modulator system constituted by the olfactory nerve fibres, mitral cells and granule cells and, moreover, there is a certain variation centred on the presence of different groups of neurons and particular neuronal connections peculiar to each class of vertebrate. Thus, in the olfactory bulb of teleosts, the great diversity of the granule cell population (Alonso, Lara, Miguel & Aijón, 1986), the existence of neuronal types not present in higher vertebrates, e.g. the ruffed cells and the perinest cells (Kosaka & Hama, 1979*a*, *b*, 1982–83; Alonso, Lara, Miguel & Aijón, 1987) and the absence of other neuronal types such as periglomerular cells or tufted cells indicate that the organisation of the olfactory bulb of teleosts is significantly different from the mammalian olfactory bulb.

The aim of this work is to study cell proliferation in the olfactory bulb of adult teleosts and to compare it with the cell proliferation described in the olfactory bulb

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and other regions of the mammalian brain. We describe the morphology, using both light and electron microscopy, of the mitotic cells and their location is compared with the distribution and characteristics of cells labelled with tritiated thymidine.

MATERIALS AND METHODS

Eight adult *Carassius auratus* (6–8 cm) obtained from commercial sources, were used for the autoradiographic study. They were anaesthetised with MS-222 (Sandoz) and subsequently injected subcutaneously with [6-^{3}H]thymidine (Amersham Buchler; specific activity 2 Ci/mMol; 5 μCi/g body weight). Each animal was given two successive injections and different survival times (2, 3, 4, and 6 days) were used. The fish were then anaesthetised and perfused through the heart using a glass pipette with the aid of a micropositioner. The brains were removed, embedded in paraffin wax and sectioned along the longitudinal and transverse planes. The sections were mounted, dipped in K2 emulsion (Ilford), stored for two months and developed in D19 (Kodak). Finally, they were counterstained by the Nissl method.

For the histological study, eight goldfish *Carassius auratus* (6–11 cm), twelve trout *Salmo gairdneri* (25–30 cm), twelve Mediterranean barbels *Barbus meridionalis* (20–30 cm), and twelve carp *Cyprinus carpio* (25–40 cm) were used. The goldfish and the trout were supplied by commercial sources, and the barbels and the carp were captured in the river Tormes (Salamanca). All the specimens were adults.

After anaesthesia with 0·03 % MS-222, six fish of each species were perfused with Bouin's fixative. The olfactory bulbs were removed and submerged in fixative, embedded in paraffin, sectioned at 10 μm along the transverse, longitudinal and sagittal planes and stained with haematoxylin–eosin or the Nissl method.

The brains of the remaining animals were perfused with a mixture of 2% paraformaldehyde–2 % glutaraldehyde in 0·16 M cacodylate buffer at pH 7·4. The olfactory bulbs were removed, stored in fixative for 1–4 hours at 4 °C, sectioned in small portions (0·5 mm) and washed with cacodylate buffer. They were postfixed in 1 % osmium tetroxide, dehydrated and embedded in Durcupan. Semithin sections (0·5 μm), stained with toluidine blue or cresyl violet, were used as controls. Ultrathin sections (40–50 nm) were contrasted with uranyl acetate and lead citrate and studied with a Zeiss EM 109 electron microscope.

RESULTS

The olfactory bulb of *Barbus meridionalis*, *Carassius auratus* and *Cyprinus carpio* is a spheroid or oval structure (Fig. 1), with a length of 1·3–1·9 mm; a short olfactory nerve enters its anterior portion and from its posterior end arises a very long olfactory tract through which it joins the telencephalic hemispheres. In *Salmo gairdneri* the olfactory bulb is a pyriform structure (Fig. 2), with a length of 1·3–1·7 mm, which rests against the telencephalon and which is joined to the olfactory mucosa by a long olfactory nerve. In all the species employed in this study the olfactory bulb shows four layers which from the outside inwards are: the olfactory nerve fibre layer, the glomerular layer, the plexiform layer and the granule cell layer (Figs 1, 2).

In the cyprinoids (barbel, goldfish and carp), towards the posterior part of the olfactory bulb there is an elongated ventricle that has a conical or pyramidal ending. In *Salmo gairdneri* no typical ventricle is present although its place is taken by a comparable structure, the posterior or ventricular recess. In these species we observed in the proximity of the ventricle or ventricular recess, the presence of an active zone

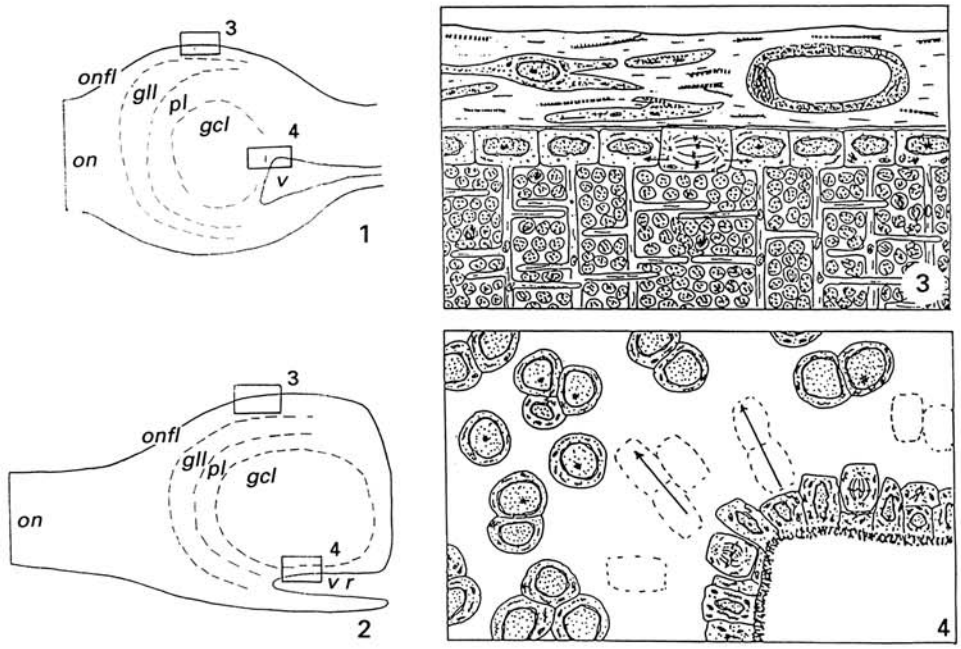


Fig. 1. Scheme of the olfactory bulb of Cyprinidae. *gll*, glomerular layer; *gcl*, granule cell layer; *on*, olfactory nerve; *onfl*, olfactory nerve fibre layer; *pl*, plexiform layer; *v*, ventricle.

Fig. 2. Scheme of the olfactory bulb of Salmonidae. *vr*, ventricular recess.

Fig. 3. Shows a higher magnification of the proliferation zone at the surface of the bulb located inside the rectangle numbered 3 in Figs. 1 and 2.

Fig. 4. Enlargement of the rectangle indicated by 4 in Figs. 1 and 2. It shows the proliferation zone near the ventricle or ventricular recess. The arrows indicate the course of migrating cells.

of cellular proliferation, composed of densely grouped cells (Figs. 7–9). In the cyprinoids, it is arranged over the most anterior part of the ventricle. It is closely related to the granule cell layer and no clear separation between the two population of cells can be seen (Fig. 5). In this zone, the cells are small with scanty cytoplasm and show a rounded or ovoid nucleus with a prominent nucleolus. [^{3}H]thymidine labelled cells are found in this area isolated between abundant unlabelled cells (Figs. 13–15). The labelled cells are located near the ventricle but clearly separate from it by one or more rows of cells. They have rounded nuclei showing different staining intensities, surrounded by a thin band of cytoplasm. In the trout the proliferation zone is extended over a broad area close to the posterior recess which shows a higher cellular density than in the cyprinoids. Moreover, the number of mitoses observed is also very low in the cyprinoids in comparison with the homologous zone in the trout. The cells that comprise it have the same characteristics as those found in the barbel and the carp. Near the lumen of the ventricular recess a relatively high number of mitoses in different phases (Figs. 7–9) can be observed. These proliferating cells are also isolated between amitotic cells as in cyprinoids.

The proliferating cells in the ventricular zone show scanty cytoplasm and are poor in organelles although some mitochondria and cisternae of rough endoplasmic reticulum arranged parallel to the plasma membrane can be seen at the cell periphery (Fig. 5). In addition, some dense zones can be seen in the plasma membrane but they do not show the typical structure of synapses.

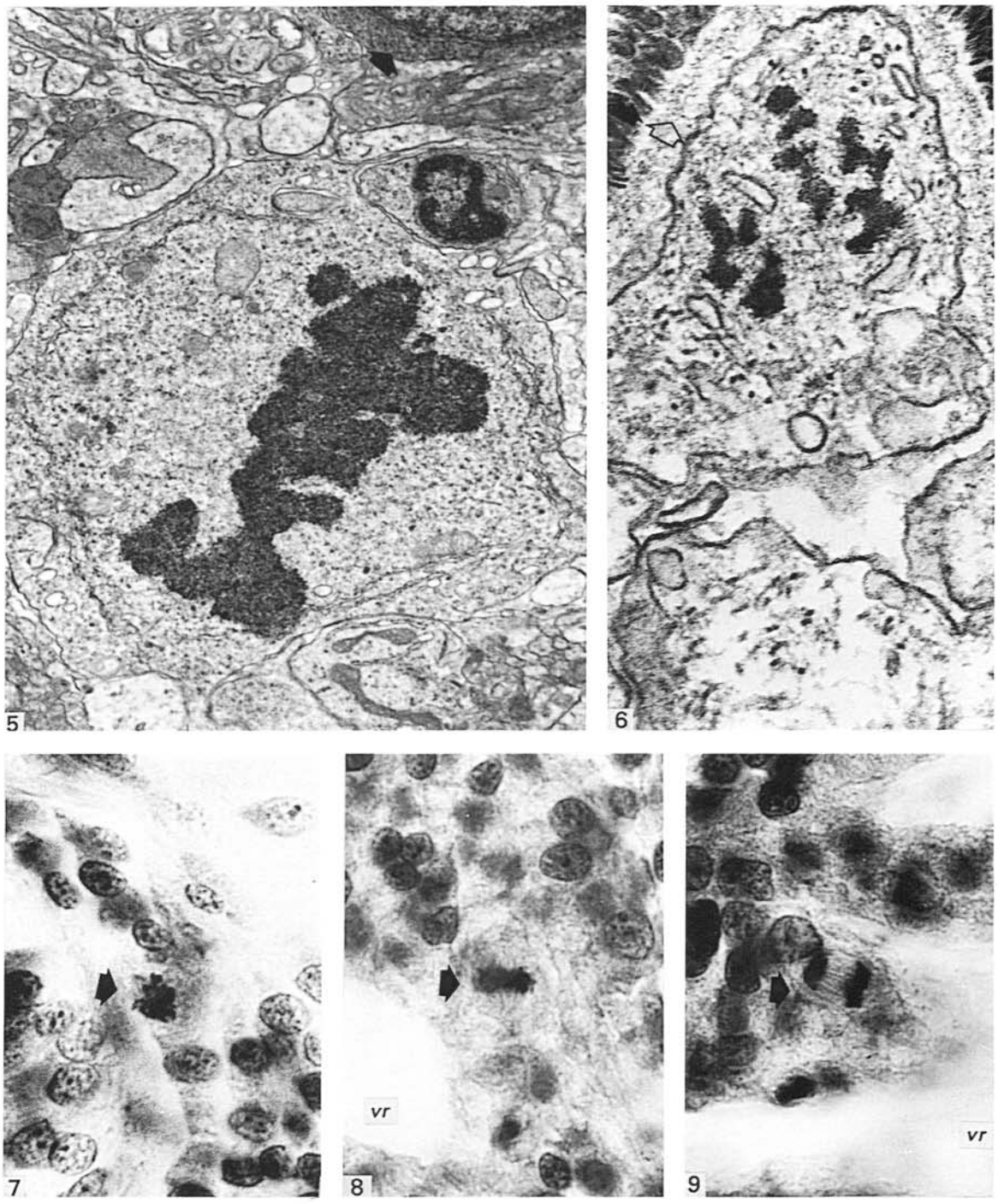


Fig. 5. Mitosis near the ventricle in *Barbus meridionalis*. The arrow points to a granule cell. ×12000.

Fig. 6. Mitosis in the peripheral zone of the olfactory bulb of *Cyprinus carpio*. The open arrow points to the basal lamina. Closed arrow, dense material adjacent to the basal lamina. ×96000.

Figs. 7–9. Cells in division in the proximity of the ventricular recess of *Salmo gairdneri*. *vr*, ventricular recess. (7) Prophase (arrow). Haematoxylin–eosin. ×600. (8) Metaphase (arrow). Haematoxylin–eosin. ×600. (9) Anaphase (arrow). Haematoxylin–eosin. ×600.

In most of the cells showing division the mitotic spindle is disposed perpendicularly to the principal axes of the ventricle (or posterior recess) and the bulb (Fig. 4), although it is occasionally orientated obliquely or parallel to these axes. Proximal to the cells undergoing mitoses it is possible to observe cells that are apparently migrating towards the granule layer.

No other such well-developed proliferation zones were observed in the olfactory bulb of the species studied. However, in the most external portion of the olfactory bulb – the olfactory nerve fibre layer – we have found a limited number of [^{3}H]thymidine-labelled cells (Figs. 10, 11) and, very rarely, mitoses (Fig. 6). In this case, the mitotic spindles seem to be orientated parallel to the surface of the bulb (Fig. 3). The mitotic cells in this zone show a very small number of organelles, e.g. ribosomes and cisternae of endoplasmic reticulum. According to their location and characteristics, they are identified as astrocytes belonging to the outer limiting glial membrane and astrocytes located between the olfactory fibres.

Between the two different proliferation zones described, we have not observed a gradient of mitoses or labelled cells. However, using the autoradiographic technique, we have found a small number of labelled cells in the glomerular, plexiform and external parts of the granule cell layer that are mostly clearly identified as glial cells, especially satellite oligodendrocytes of mitral or ruffed cells (Fig. 12).

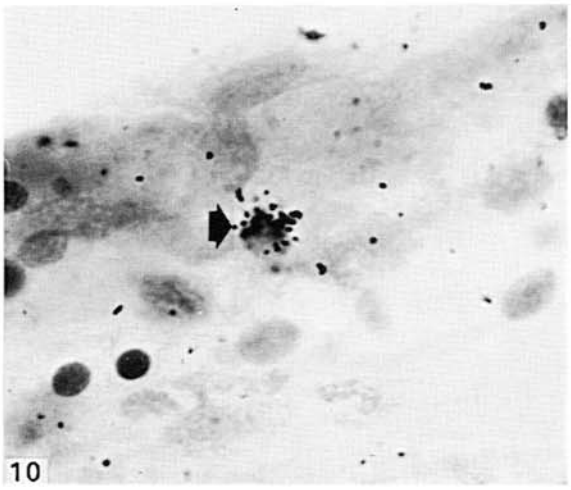
DISCUSSION

The existence of a limited cell proliferation appears to be a normal feature of the brain of some adult mammals (Bryans, 1959; Kaplan & Hinds, 1977). Continuous growth has been reported in the brain of teleosts (Bauchot, Platel, Ridet & Thireau, 1973) with production of new cells, at least for an extended period into adult life (Meyer, 1978; Raymond & Easter, 1983). The area of the brain in teleosts in which cellular proliferation has been extensively studied, both in juvenile and adult periods, is the optic tectum. Thus, it has been examined under different experimental conditions (Johns *et al.* 1977; Kirsche & Kirsche, 1961; Raymond *et al.* 1983; Stevenson, 1976, 1977; Stevenson & Yoon, 1978), and its production of neurons and glia during normal growth has been described (Raymond & Easter, 1983). However, no data are available on the cellular proliferation in the teleost olfactory bulb.

Mitoses have been described in the central nervous system of mammals with two characteristic locations: ventricular (Allen, 1912; Altman, 1969; Bryans, 1959) and peripheral, the latter being located deep inside the wall of the cerebral hemispheres (Zamenhof, 1985). Mitotic cells in the ventricular location could differentiate into both glia and neurons or they could be neuroblasts proliferating at specific times and locations and possessing specific receptors for specific mitogens. The significance of the deep mitoses is not clear, although it has been suggested that they could be glioblasts, in particular precursors of astrocytes (Rakic, 1981–82) or they may be mitoses of endothelial cells (Zamenhof, 1985).

The presence of cell proliferation in the ventricular zone of the mammalian brain agrees well with our own observations in the same zone of the olfactory bulb of freshwater teleosts. However, the other proliferation zone described in our material has not been previously described in the olfactory bulb of mammals.

Regarding the nature of the cells undergoing division that are observed in the vicinity of the ventricle (or posterior recess), our results suggest that these cells are not glial elements but they can be identified as granule cells or precursors of granule cells. Indeed, the cells have not the irregular profiles of the protoplasmic astrocytes and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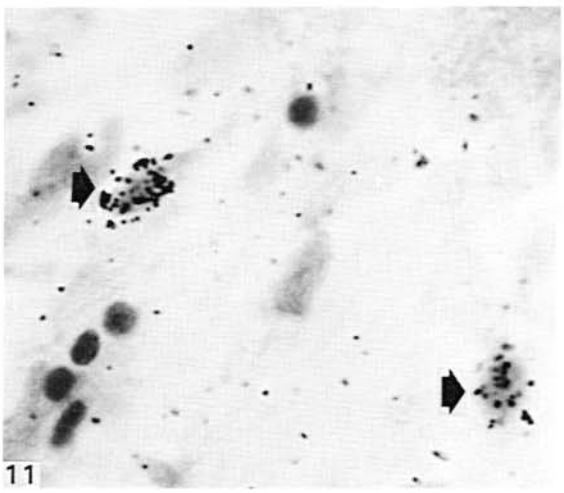
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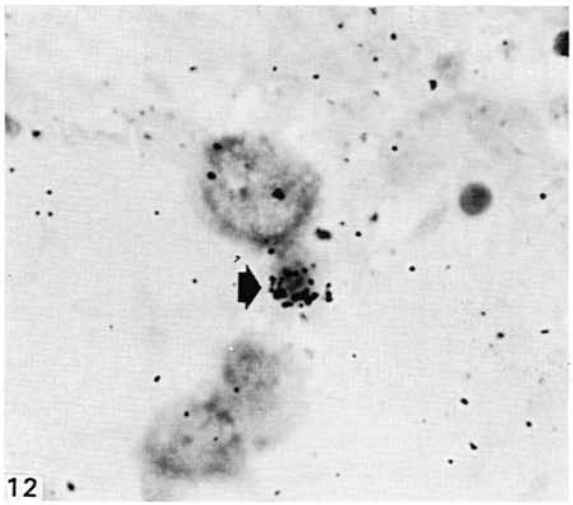
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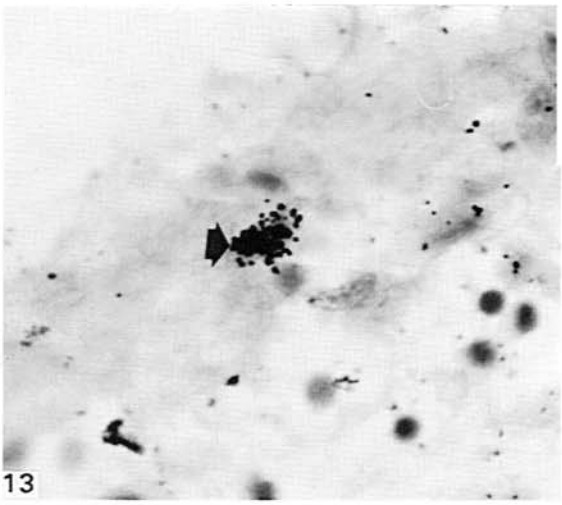
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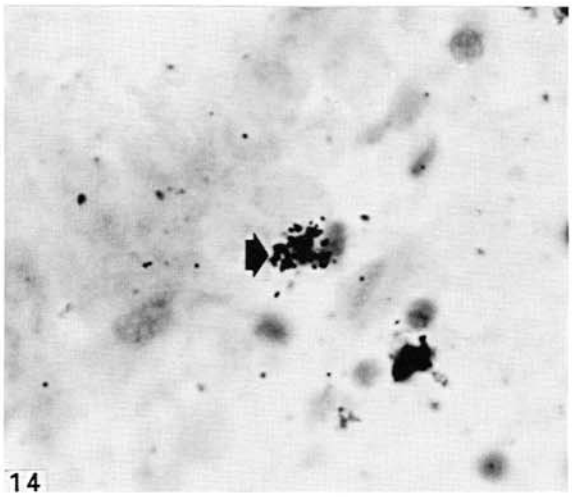
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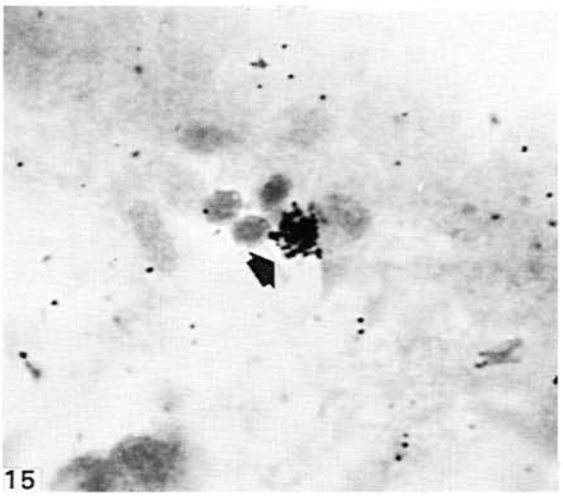
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microglia, neither do they exhibit the fasciculi of microfilaments of the fibrous astrocytes, nor does their cytoplasm show the electron density of oligodendrocytes and microglia. In addition, their location, small size and morphology coincide with the characteristic features that have been reported concerning the granule cells (Price & Powell, 1970). Moreover, the findings of Altman (1963) and Hinds (1968*b*) support such identification since these authors showed that most of the granule cells of the olfactory bulb are formed during the postnatal period. However, Kaplan & Hinds (1977) showed DNA synthesis in cells that seem to be small granule cells in the olfactory bulb of adult rats. In addition, the presence of cells apparently migrating, which in *Salmo gairdneri* are clearly observable, also suggests neuron production (Gruberg & Stirling, 1974).

On the other hand, the cells in division at the surface of the olfactory bulb show a lower mitotic frequency in our study in comparison with those in the ventricular region. Deep or peripheral mitoses have been described in the cerebral hemispheres of fetal rats (Zamenhof, 1985, 1987). Different hypotheses have been proposed about the nature of the cells originating from these deep mitoses (Rakic, 1981–82; Zamenhof, 1985). Our results demonstrate that they are marginal astrocytes and astrocytes located between the olfactory fibres. Their location is clearly defined by the presence of the basal lamina and the osmiophilic dense material previously reported by us (Lara, Alonso, Miguel & Aijón, 1987). Such astrocytes could correspond to a type of poorly differentiated astrocytes described by Braak (1975) in the human glial stratum.

Regarding the possible function of the cellular proliferation in adult life, Altman (1967) suggested that the production of microneurons may be responsible for neural 'plasticity' or may be the substrate of memory. Thus, the high neurogenesis observed in *Salmo gairdneri* could correlate with the excellent olfactory memory represented in the homing behaviour of numerous salmonids. On the other hand, Bayer (1986) points out that the production of new granule cells in the olfactory bulb does not result in a population increase of these cells and must therefore act as a mechanism for cell turnover. Thus, prolonged neurogenesis in the olfactory bulb may be more suggestive of cell turnover in a numerically stable population than of cell accumulation in a growing population. In support of this possibility is the observation in this zone, in all the species studied by us, of some cells undergoing karyolysis under apparently normal conditions. Moreover, preliminary findings on cell counts and volumetric variations of the granule cell layer in the olfactory bulb of freshwater teleosts (unpublished results) reveal that the cell population does not show any significant variations.

The deep cell proliferation as shown by the labelled cells in the glomerular and plexiform layers seems to represent normal gliogenesis (Rakic, 1981–82; Zamenhof, 1985). The divisions of the labelled cells in the olfactory nerve fibre layer could represent the response to the continuous growth of the adult olfactory bulb in the teleosts, providing the corresponding increase in surface area. However, another possibility could be that the division of these astrocytes represents the reaction to changes and regeneration in the neuronal population of the olfactory mucosa.

Figs. 10–15. Labelled cells with tritiated-thymidine in the olfactory bulb of *Carassius auratus*. (10) Marginal astrocyte (arrow) in the olfactory nerve fibre layer. ×350. (11) Astrocytes (arrows) between olfactory fibres. ×350. (12) Satellite oligodendrocyte (arrow) of a ruffed or mitral cell. ×350. (13) Granule cell or precursor of granule cell (arrow). Ventricular zone. ×350. (14) Granule cell or precursor of granule cell (arrow). Ventricular zone. ×350. (15) Granule cell or precursor of granule cell (arrow). Ventricular zone. ×350.

In conclusion, comparing the cell proliferation in the olfactory bulb of adult mammals and adult teleosts, we can conclude that the latter group shows a higher activity than the first, but produces predominantly granule cells. However, we have observed a second zone of proliferation in the surface of the olfactory bulb with a lower cell production, clearly related to the new formation of astroglial cells. This second zone has not been previously described in the vertebrate olfactory bulb and could be a specific characteristic of the cell proliferation in the teleost brain.

SUMMARY

The olfactory bulb of adult specimens of *Carassius auratus* was subjected to an autoradiographic study using [^{3}H]thymidine. Adult specimens of *Carassius auratus*, *Barbus meridionalis*, *Cyprinus carpio* and *Salmo gairdneri* were also studied using light and electron microscopy. Cells were found undergoing mitotic division with two characteristic locations: a group can be seen near to the ventricle or ventricular recess; these cells can be identified as granule cells or precursors of granule cells. Other cells undergoing division are seen on the outer limiting glial membrane and in the olfactory nerve fibre layer; these cells are identified as astrocytes. Different hypotheses about the destination and meaning of these proliferating cells and the similarities and differences with the cell proliferation in adult higher vertebrates are discussed.

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