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THE OUTER FIBRILLARY LAYER IN THE OLFACTORY BULB OF FRESHWATER TELEOSTS

BY

J. R. ALONSO, J. LARA, R. COVEÑAS, E. VECINO, C. PIÑUELA and J. AIJON

(Cytology and Histology, Department of Cell Biology, Faculty of Biology, University of Salamanca, Salamanca, Spain)

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ABSTRACT. — *The outer fibrillary layer in the olfactory bulbs of the teleosts is composed by the olfactory fibres, several types of glial cells, displaced neurons, and neurons and fibres from the terminal nerve. The organization of this stratum is partially different from that in the mammalian olfactory bulb.*

Key words : Olfactory fibre, olfactory bulb, teleost, glia, cytoarchitecture.

INTRODUCTION

The olfactory bulb constitutes the first relay station in the olfactory pathway in all vertebrates. The axons of the bipolar neurons of the olfactory mucosa course through the olfactory nerve and reach the bulb where they are distributed over the periphery, forming the first stratum of the bulbar lamination, the outer fibrillary layer or olfactory nerve fibre layer.

The organization of the olfactory bulb in teleosts is clearly different from that of mammals. Thus, the lamination is distinct (ALONSO *et al.*, 1988d), there are different projection pathways (LEVINE and DETHIER, 1985; BARTHELD and MEYER, 1986) and new neuronal types have been described : ruffed cells (KOSAKA and HAMA, 1979a-b, 1980, 1982-83; ALONSO *et al.*, 1987b), perinest cells (KOSAKA and HAMA, 1982-83), displaced granule cells (ALONSO *et al.*, 1987a), mixed-synapse cells (KOSAKA and HAMA, 1982-83),..

Neuronal and glial proliferations in both the internal and peripheral zones have also been reported in the olfactory bulb of adult fish (ALONSO *et al.*, 1988c). Moreover, differences in the glial typology in the outer fibrillary layer has been described under normal conditions in the rat (FAUCETTE, 1984). Finally, we have recently observed differences in the immunoreactivity of the olfactory fibres between teleosts and higher vertebrates (unpublished observations).

All these characteristics — changes in the neuronal and circuitry organization, interspecific differences, cell proliferation and the peculiar characteristics of the glial cell population, presence of different neuroactive substances or neurotransmitters — point to the need for new investigation centered on the outer fibrillary layer of the teleostean olfactory bulb, comparing these observations with previous reports in higher vertebrates.

We therefore carried out a study with both light and electron microscopy of this stratum in five species of freshwater teleosts belonging to three different orders.

MATERIALS AND METHODS

In the present study 9 specimens of *Tinca tinca,* 6 specimens of *Barbus meridionalis* and 9 specimens of *Cyprinus carpio* (Or. Cypriniformes), 11 specimens of *Salmo gairdneri* (Or. Salmoniformes) and 8 specimens of *Gambusia affinis* (Or. Cyprinodontiformes) were used.

After anaesthesia with tricaine methanesulphonate (MS-222, Sandoz) at 0.03% (0.01% for *Gambusia affinis),* the animals were perfused with 0.63% saline followed by a mixture containing 1% paraformaldehyde and 1% glutaraldehyde in 0.12M phosphate buffer (pH 7.3). In the case of *Gambusia affinis,* owing to the difficulty in perfusing the animals, the brain was fixed by immersion and then embedded, thereafter following protocol described for the other species.

Three animals from each species were used as controls for light microscopy. The olfactory bulbs were extracted, embedded in Paraplast and serially sectioned at 10 μm along the longitudinal, transversal and sagittal planes. They were stained with hematoxylin-eosin or following the Nissl technique.

The olfactory bulbs of two animals were used for Golgi impregnation. After fixation, the olfactory bulbs were immersed in

a freshly prepared solution of osmium-dichromate (0.2 g osmium tetroxide and 2.4 g potassium dichromate in 100 ml distilled water) for three days, after which they were transferred to 0.75% silver nitrate for two days. Following this, 100-150 μm horizontal and sagittal sections of the olfactory bulbs were cut serially with a vibratome (Campden Instruments). Non impregnated sections were inserted between pieces of Parafilm, covered with 5% agar and subjected to a second Golgi impregnation as described previously (ALONSO *et al.*, 1988b).

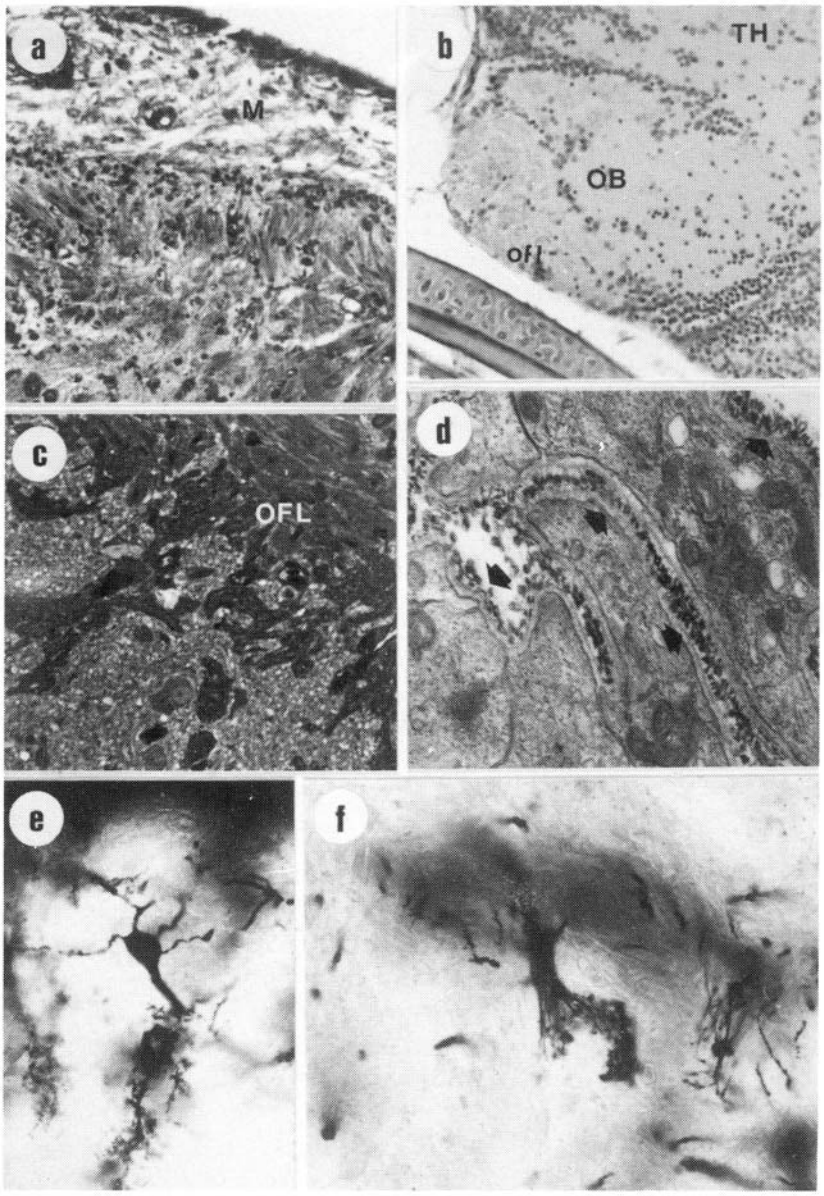
The remaining animals were used for electron microscopy. After perfusion, the olfactory bulbs were cut into small portions and washed in the same buffer with 10% sucrose, postfixed for 1 hour with 1% osmium tetroxide in phosphate buffer and washed again with phosphate buffer. After dehydration in a graded ethanol series, adding 1% uranyl acetate during the 70% ethanol phase, followed by two washed in propylene oxide for 20 minutes, each of the pieces was embedded in Spurr resin.

Using an Ultracut E (Reichert-Jung) ultratome, semithin sections (0.5-1 μm) were obtained which, after staining with toluidine blue or Ziehl's fuchsin (ALONSO *et al.*, 1988a), were used for light microscopy and were kept as controls for electron microscopy. Ultrathin section (40-50 nm) were contrasted with uranyl acetate and lead citrate for study under a Zeiss EM109 electron microscope.

RESULTS

The presentation of the findings will focus on the general organization of the olfactory fibres which constitute the Outer Fibrillary Layer; the ultrastructural characteristics of these axons, the glial cells and prolongations present in this stratum and, finally, the unknown presence of neuronal elements on it.

There are some differences in the structure of the olfactory bulb in the species studied. Thus, in the cypriniforms the olfactory nerve is very short, composed of different identifiable fibrous bundles which enter the bulb forming a more or less concentric stratum (Fig. 1a). In the salmoniforms, the olfactory nerves are very long, compact, and their implantation cone is very marked. Their fibres extend throughout the periphery of the olfactory bulb, but specially on its rostral and dorsal portions. Finally, in the cyprinodontiforms,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the olfactory nerves are long and curved and their fibres are distributed only through the anterior and ventral portions of the olfactory bulb (Fig. 1b). However, in spite of these differences in the development and arrangement of the Outer Fibrillary Layer, its components and ultrastructural organization are similar in all the species studied.

Two clearly different components were observed in the olfactory nerve of the species studied : the olfactory axons forming compact bundles of fibres (Fig. 2a), and the Terminal Nerve.

The olfactory axons, originated from the bipolar neurons of the olfactory mucosa, are thin amyelinic fibres (Figs. 2a, 3a) which penetrate the bulb through its rostral face, forming thick bundles with a variable number of fibres (Fig. 1f). These latter contain 4-10 microtubuli, some cisternae of smooth endoplasmic reticulum and, occasionally, mitochondria occupying the whole profile of the fibre (Figs. 2a, 2d, 3).

The fibrous bundles are surrounded by astrocytic processes with a scanty and very weakly electron-dense cytoplasmic content (Figs. 2a, 2d, 3a, 3b). Occasionally, small junction structures are observed between two fibres of the same bundle (Fig. 2e). In the zone where the fibres reach the olfactory bulb, some are seen to penetrate directly into deeper layers though most are distributed over the bulbar surface, forming the first of the layers composing it; the outer fibrillary layer (OFL) or olfactory nerve fibre layer (Fig. 1c). The olfactory fibres can be followed using the Golgi technique and no ramifications of the olfactory axons are observed in this stratum (Fig. 1f). Thus, there are no olfactory fibre taking part in the formation of more than one glomerulus.

Light and electron microscope techniques permitted the distinction of two sublayers within the OFL according to the trajectory of their fibres and the number and type of cells contained in them

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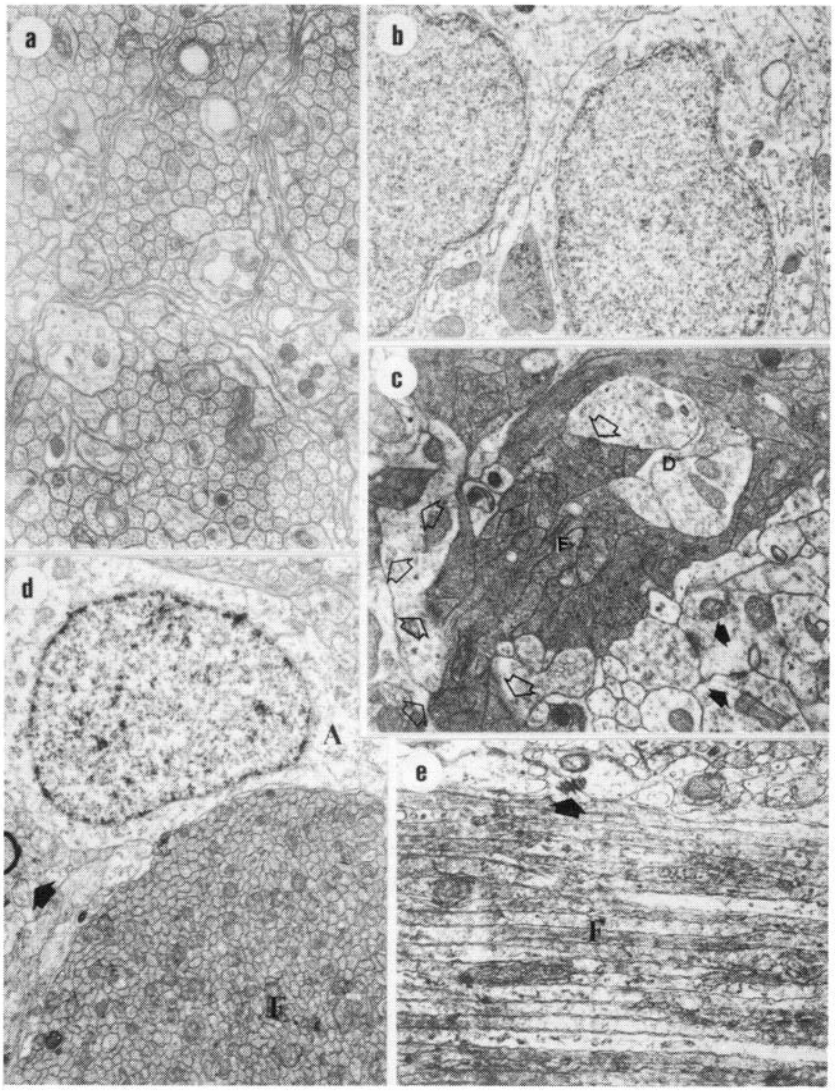
FIG. 1.

a. Transversal section of the olfactory bulb of *Salmo gairdneri*. Note numerous cells in the outer sublayer. (M : meninx). Hematoxylin-eosin. 400×.
b. Longitudinal section of the olfactory bulb of *Gambusia affinis*. (OB : olfactory bulb; ofl : outer fibrillary layer; TH : telencephalic hemisphere). Hematoxylin-eosin. 125×.
c. Semithin section stained with Ziehl's fuchsin. *Tinca tinca*. 500×.
d. Dense osmiophilic material (arrows) in the surface of the olfactory bulb of *Barbus meridionalis*. 20000×.
e. Impregnated astrocyte in the outer glial limiting membrane. *Barbus meridionalis*. 1200×.
f. Bundle of olfactory fibres entering into the glomerular layer. *Salmo gairdneri*. 400×.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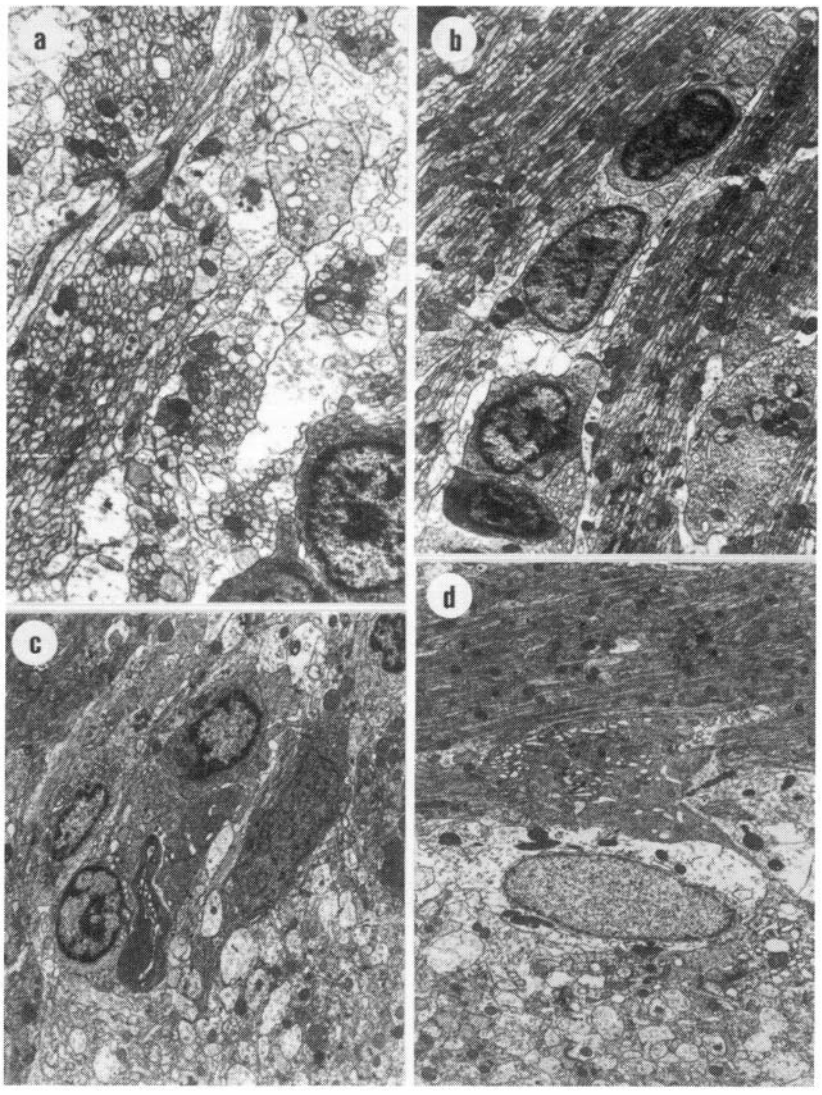
(Fig. 1a). In the external sublayer the fibres follow a course parallel to the surface of the bulb and its cells are abundant, whereas in the inner sublayer the fibres follow a course which is oblique or perpendicular to the bulbar surface and its cells are less numerous. Differences are also found between the cell types in each sublayer.

Between the cells observed in the OFL, extravasate blood cells, mostly lymphocytes, are a common finding. Among the glial cells, of outstanding interest are the astrocytes which form the outer limiting glial membrane. With control techniques, numerous small cells with a dense nucleus are observed to form a band on the limit of the olfactory bulb (Fig. 1a). Silver impregnation techniques reveal that some of these are astrocytes featuring pyriform cell body with a long highly-ramified prolongation coursing towards the interior and a further four or five much finer and unbranched prolongations with bulbous endings (Fig. 1e). These prolongations are situated between the fibres and follow their trajectory. According to electron microscopy, these cells have an oval or slightly irregular nucleus, with finely disperse chromatin (Fig. 2b). Their cytoplasm is scarce and very weakly electron-dense, containing a few organelles. No or very scarce fascicles of gliofilaments are observed. Apart from these "normal" astrocytes (Fig. 3d), we also observed other cells which we think are also astrocytes according to their characteristics and their position between the fascicles of fibres among which their prolongations are arranged. They also have scarce cytoplasm, small mitochondria, and isolated cisternae of endoplasmic reticulum, but their nuclei are very heterochromatic (Fig. 3b). These differences in the chromatin content of the nucleus can be related with the presence of mitotic astrocytes or precursors observed in the same region (ALONSO *et al.* 1988c). There are also interfascicular oligodendrocytes situated in rows between the fibrous bundles (Figs. 2b, 3c).

FIG. 2

a. Transversal section of olfactory fibres and astrocytic processes in the cyprinoid olfactory bulb. 20000×.
b. Astrocytes of the outer glial limiting membrane. *Salmo gairdneri.* 20000×.
c. Olfactory fibre endings in the glomerular layer. Note the electron-dense content of the olfactory fibres (F). Open arrows : axo-dendritic synapses between olfactory fibres and mitral cells dendrites (D). Arrows : dendro-dendritic synapses. *Tinca tinca.* 20000×.
d. Astrocyte (A) surrounding a large bundle of fibres (F). Arrow points to a myelinated fibre in the same region, clearly larger than the olfactory fibres. *Barbus meridionalis.* 12000×.
e. Junction structure in the outer fibrillary layer (F : fibres). *Salmo gairdneri.* 20000×.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Another characteristic of the external sublayer is the existence of a dense osmiophilic material, with a bead-like appearance, located externally to the basal lamina of the outer limiting glial membrane of the olfactory bulb which we have observed in the Cypriniform species (Fig. 1d). This material follows an irregular course along the highly interdigitated zone between the outer limiting glial membrane and the pia surrounding the olfactory bulb. Thus, these structures display a complex structure, suggesting a strong resistance.

The innermost sublayer of the OFL has few cells. Among these, outstanding are neurons in a "displaced" position such as mitral cells, ruffed cells and perinest cells. These neurons were easily identified using the Golgi method. With light and electron microscopy, these cells shows the same morphological characteristics as those belonging to the cell type in the non-displaced position. The fibres of this sublayer course obliquely or perpendicularly to the surface of the bulb. The fascicles are smaller than those observed in the external sublayer, probably owing to the division of some of them (Figs. 2c, 3c, 3d).

Finally, in the OFL of the species studied we observed the presence of very large neurons, occasionally forming dense clusters of cells; these were identified as ganglionar cells of the terminal nerve. The fibres of this nerve run together with the olfactory fibres; however, it constitutes a mainly independent system.

Discussion

The Outer Fibrillar Layer is present in the olfactory bulb of all vertebrates except the Amphioxus (Ramon y Cajal, 1911; Allison, 1953). We have described significant interspecific differences in this stratum (Alonso *et al.*, 1988d). Thus, *Gambusia affinis* shows a lower degree of development of this layer, which is highest in *Salmo gairdneri*. The more or less rostral location of the

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Fig. 3.
Panoramics of the OFL showing bundles of fibres and different types of glial cells.
a. *Gambusia affinis*. 16000×.
b. *Tinca tinca*. 12600×.
c. *Barbus meridionalis*. 12600×.
d. *Barbus meridionalis*. 12600×.

olfactory bulbs seems to be unrelated with the extent and organization of the OFL. Compared with higher vertebrates, there are also important differences in the presence of displaced neurons which were not observed in mammalian olfactory bulbs.

The olfactory fibres arrive and arborize in the OFL of teleosts, as has been described in higher verterbrates (RAMON Y CAJAL, 1911). ANDRES (1970) has reported several differences between the fascicles and olfactory fibres and similar morphological observations are described in our results. Thus, ANDRES (1970) has mentioned large variations in the number of microtubuli which, according to this author, may allow types of bipolar neurons in the olfactory mucosa responding to different primary odors to differentiate. We also observed similar variations between various fibrous bundles according to the number of microtubuli in their axons. In a similar way, the fibres which we observed in transversal sections showing only endoplasmic reticulum are identified as regenerating axons from the mucosa (ANDRES, 1970).

The dense osmiophilic material which we have observed in the surface of the cyprinoid olfactory bulbs seems to be also a peculiar structure of the olfactory bulb of teleosts (LARA *et al.*, 1987), unreported in higher vertebrates.

Although morphologically the olfactory fibres do not display a differential arrangement, a profuse crossing of fascicles — specially in the inner sublayer — being observed, there must be some topological distribution. The possibility of such a topological organization between the olfactory mucosa and the olfactory bulb was reported by ADRIAN (1951) and LEGROS CLARK (1951, 1957) in mammals. The latter author, using retrograde degeneration, observed in the rabbit that the dorsal portions of the epithelium project to the dorsal regions of the olfactory bulb and similarly for the ventral zones. The evidence from electrophysiological (ORREGO, 1961, LEVETEAU and MACLEOD, 1976), metabolic (SHARP *et al.*, 1975) and anatomical studies (LAND *et al.*, 1970; PEDERSEN *et al.*, 1986) suggests that different groups of primary axons, from different zones of the olfactory mucosa, project to particular glomeruli in the bulb. In teleosts, THOMNESEN (1978), using *Salmo trutta* and *Salvelinus alpinus,* has found that on exposing the mucosa to different odorants the potentials evoked were different among the same portions of the olfactory bulb. Similar results, but using different aminoacids, were observed in the olfactory bulb

of *Carassius auratus* (MEREDITH, 1974) and *Salmo gairdneri* (MACLEOD and LOWE, 1976). These results demonstrate that the principle of spatial separation for the different odors in the olfactory fibres is valid not only in macrosmatic mammals, but also in teleosts. Moreover, physiological data (SATOU *et al.*, 1983) suggest a differentiation between the medial and lateral portions of the olfactory bulb which have been related, respectively with the medial and lateral fascicles of the olfactory nerve and which project through the medial and lateral olfactory tract to different terminal fields in the telencephalic hemispheres. The presence, described in the present report, of junction structures between two fibres of the same bundle seems to be also a peculiarity of the olfactory bulb of teleosts, not being previously described.

The glial cells of the outer glial layer were early identified as astrocytes (RAMON Y CAJAL, 1911). These cells have the typical astrocytic appearance with long processes directed towards the first neural layer (BONDAREFF and MCLONE, 1973). However, the morphology of these cells, as we have described using Golgi impregnation, shows a high degree of differentiation with undivided prolongations forming a palisade parallel to the surface of the bulb and long prolongation highly ramified surrounding the olfactory fibres. Later, the identification of these cells of astrocytes was confirmed by electron microscopy. However, the majority of these studies have been done in mammals : RAMSEY (1965) and BRAAK (1975) in man, BRIGHTMAN and REESE (1969) and HATTON and ELLISMAN (1981) in the rat, JONES (1970) in the cat and BONDAREFF and MCLONE (1973) in the monkey, RAINE *et al.* (1978) in the guinea pig, MCLONE (1980) in the mouse and SUAREZ and FERNANDEZ (1983) in the hamster, the data in lower vertebrates being very scarce. We consider that our description offers new data on the characteristics of this glial type in different families of freshwater teleosts. The variations which we observed in the glial population of the OFL may be related with the presence of new olfactory axons after the continuous cell renewal observed in normal and damaged olfactory epithelium of adult animals (GRAZIADEI and MONTI GRAZIADEI, 1978, 1979).

In the monkey, WOLFF (1970) has reported the presence of collagen fibres and other connective components joining the glia limitants and the connective envelope as we have observed in our material. On the other hand, we consider that in teleosts these structures displayed a high degree of interrelation and interdigitation.

The ordering of glial cells in rows between the axonal fascicles could be due to the high density of axonic bundles in this zone. Thus, the astrocytes do not only surround the olfactory axons but they are also packed by the fibres. Another possibility is that the row-like arrangement would be the result of cell proliferation from a single precursor cell. However, the coexistence observed of different cell types (e.g. astrocytes and oligodendrocytes) in the same row should also be taken into account.

ARIENS-KAPPERS *et al.* (1936) have mentioned the presence in mammals of traces of myelin in connection with the olfactory fibres. We observed no myelinated fibre and consider their existence improbable due to the glial cells observed in the olfactory nerve. We believe these myelin traces could be related with the fibres of the Terminal Nerve. Fibres of the Terminal Nerve with a thin myelinic sheath have been described by JOHNSTON (1913) in the horse.

Finally, the neurons observed in the inner sublayer of the OFL, would be a characteristic situation of lower vertebrates, not observed in higher vertebrates due to the clearer lamination of the olfactory bulb observed in the latter group, specially in macrosmatic mammals, which have received the most attention. We consider these neurons as displaced cells because their morphological characteristics coincide with those observed and described for these cell types.

SUMMARY

The outer fibrillary layer (OFL) is the outer-most stratum in the olfactory bulb of all vertebrates. We have studied the structure and ultrastructure of this layer in five species of freshwater teleosts, using light and electron microscopy. There are differences in the development and arrangement of the OFL between the species studied; however, its components and ultrastructural organization are similar in all of them. The elements of the OFL were identified as small unmyelinated olfactory fibres forming dense bundles, different types of glial cells, fibres and gangliona cells from the terminal nerve, and displaced neuronal elements, such as mitral cells or ruffed cells. Some variations of glial cells and the displaced neuronal elements were not observed in the mammalian OFL. These observations indicate some peculiarities of the teleostean OFL

suggesting an organization of this stratum partially different from previous descriptions on higher vertebrates.

Résumé

La couche fibrillaire externe est la strate la plus externe dans le bulbe olfactif chez tous les vertébrés. Nous avons étudié la structure et l'ultrastructure de cette couche chez cinq espèces de téléostéens d'eau douce, en utilisant des techniques pour la microscopie optique et électronique. Il y a des différences dans le développement et la disposition de l'OFL entre les espèces étudiées. Néanmoins ses composants et l'organisation ultrastructurelle est similaire chez les espèces étudiées. Les éléments dans l'OFL sont des petites fibres olfactives amyélinisées disposées en paquets denses, divers types de cellules gliales, des fibres et des cellules ganglionaires du nervus terminalis, et des éléments neuronaux déplacés (cellules mitrales et cellules "ruffed"). Quelques variantes des cellules gliales et les éléments neuronaux déplacés ne sont pas observés dans l'OFL chez les mammifères. Ces observations indiquent quelques particularités de l'OFL chez les téléostéens, et suggèrent pour cette structure une organisation partiellement différente des descriptions chez les vertébrés supérieurs.

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Dr. J. Aijón
Citología e Histología
Departamento de Biología Celular
Facultad de Biología
37008 Salamanca
Spain