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Ruffed Cells in the Olfactory Bulb of Freshwater Teleosts
II. A Golgi/EM study of the ruff

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With 5 Figures

Summary: The morphological characteristics of the synaptic contacts in the ruff of the cichlid fish *Hemichromis bimaculatus* were studies using the combined Golgi-electron microscope technique. Ruffed cells were located in the glomerular and plexiforme layers and exhibited a pyriform or round cell body and numerous thin dendritic branches that were highly ramified. Four different segments could be distinguished on the initial portion of the axon (IP) according to the number and density of protrusions. These protrusions or lateral appendages are highly interdigitated, forming a characteristic synaptic field: the ruff. The ruff displayed a very high number of synapses with terminals showing a varied morphology. Protrusions of the ruff were both presynaptic and postsynaptic, taking also part in reciprocal pairs of synapses. Synapses from the ruff to the adjacent prolongation are asymmetrical, the prolongation to protrusion synapses being symmetrical. The axonal shaft participates in fewer synaptic contacts. Boutons contacting with one protrusion can synapt with other one, and can also receive an asymmetric synapse from another terminal, forming a serial synapse. This constitutes the most complex synaptic system observed in the glomerular layer of the olfactory bulb in any vertebrate. The synaptology of the ruffed cell IP is compared with previous reports on other species, with the teleostean mitral cells and with the IP of higher vertebrates neurons, the ruffed cells showing a completely different synaptic pattern.

Key Words: Ruffed cell, synapse, olfactory bulb, Golgi/EM, teleost

Introduction

The structure of the teleostean olfactory bulb shows important differences with that of mammals (Alonso et al., 1988). One such difference is the presence in teleosts of a new neuronal type, the ruffed cell, whose distinctive characteristic is the presence on the initial portion of the axon (IP) of a series of highly interdigitated protrusions that form an ovoid field, the ruff (Kosaka, 1980; Kosaka and Hama, 1979a, b, 1980, 1982—83; Alonso et al., 1987). These protrusions are more prominent and complex than other modifications of the IP previously described and seem to play an important role in the modulation of the sensory input in the teleost olfactory bulb.

Hitherto, the ruffed cell has not been described in the olfactory bulb of other classes of vertebrates. Golgi studies have demonstrated the presence of this cellular type in several orders of teleosts, showing variations in the number, kind and branching pattern of the dendrites and in the disposition of the protrusions in the IP (Kosaka and Hama 1980; Alonso et al., 1987). However, electron microscope observations are relatively scarce and have only centered on three species whose IP and dendritic arborizations show significant differences. In order to gain a better understanding of the connections of this strongly modified IP, we examined in serial sections the complex structure of the ruff in a new species, *Hemichromis bimaculatus*, phylogenetically distant from species examined in other reports. The irregular protrusions of the ruff along the serial sections were identified using the combined Golgi/EM technique.

Materials and Methods

Three adult male specimens of *Hemichromis bimaculatus* (Family Cichlidae) kept under standard laboratory conditions (12/12 hours light-dark cycle) were used in the present study. The animals were anaesthetized with tricaine methanesulfonate (MS-222, Sandoz) at 0.03% and transcardially perfused with 15 ml of 0.63% saline followed by 250 ml of a mixture containing 1% glutaraldehyde and 1% paraformaldehyde in 0.12 M phosphate buffer, pH 7.3. After perfusion, the fish were kept in the refrigerator overnight. The following day, the olfactory bulbs were dissected out and processed according to a slightly modified version of the combined Golgi/electron microscope procedure of Fairen et al. (1977). Briefly, the olfactory bulbs were immersed in a freshly prepared osmium dichromate solution (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. Thereafter, 50 μm horizontal sections of the olfactory bulbs were cut serially with a vibratome. Non impregnated sections were

piled between pieces of Parafilm, covered with 5% agar and subjected to a second Golgi impregnation as described previously (FROTSCHER and ZIMMER 1986; ALONSO et al., 1988).

Sections containing well-impregnated ruffed cells were photographed and drawn. They were then subjected to the goldtoning procedure, including immersion on 0.05% gold chloride followed by 0.05% oxalic acid, and a bath of sodium thiosulfate to remove the silver chromate precipitate from the original Golgi impregnation. The sections were post-fixed in osmium tetroxide, dehydrated (block stained with 1% uranyl acetate in 70% ethanol) and embedded in Araldite between aluminum and plastic foil. Ultrathin serial sections of selected ruffed cells were cut on a Reichert Jung Ultracut E ultrotome, after the cells had been reembedded into plastic capsules and had been closely trimmed. The ultrathin sections were mounted on Formvar-coated slot grids and studied in a Zeiss EM 10 or a Zeiss EM 109 electron microscope.

Results

Ruffed cells from *Hemichromis bimaculatus* were found at different levels in the glomerular layer of the olfactory bulb. Using light microscopy, these cells exhibited round or ovoid cell bodies from which arose one or more thick dendritic trunks (Figs. 1, 2). These dendrites branched and intermingled with each other, giving rise to numerous small thin prolongations. The axons of the ruffed cells frequently arose from the pole opposite to the dendrites showing
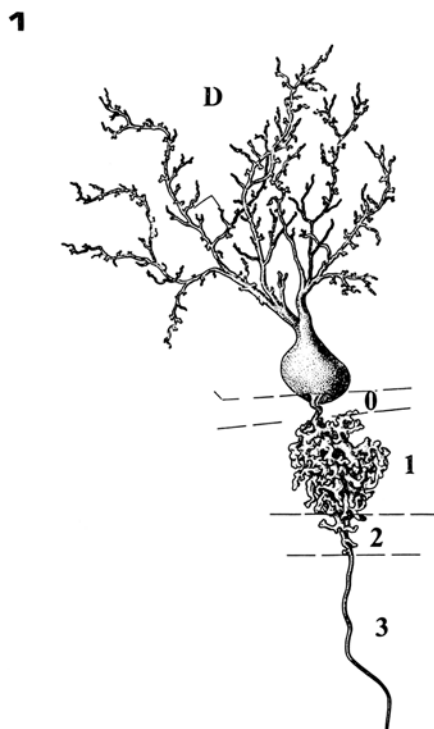

Fig. 1. Camera lucida drawing of a Golgi-impregnated ruffed cell. The initial portion of the axon may be subdivided into four different segments ("0", "1", "2" and "3") according to the number and disposition of the axonal protrusions. (D: dendritic arborization)

numerous elaborated protrusions around a central shaft (Figs. 1, 2). Four different portions could be distinguished in the IP of the ruffed cells of *Hemichromis bimaculatus* according to the number, length and disposition of the protrusions along the shaft (Fig. 1). Thus, the first part, or segment "0", extended for a short distance (3—6 µm) between the cell body and the ruff and did not contain protrusions (Fig. 4 A). The second part or segment "1" displayed numerous protrusions around the axonal shaft, forming an ovoid field: the ruff. The large protrusions gave off new branches and normally ended in short terminal knobs. Segment "2" showed smaller isolated protrusions, extending for a very short distance. The shaft was clearly observable. The last portion or segment "3" was smooth, slightly undulated and extend for a very long distance until its impregnation suddenly ended.

At electron microscope level, the axonal shaft displayed typical IP characteristics: a layer of dense granule material forming an undercoating, fascicles of microtubules and scattered clusters of ribosomes. Other organelles such as mitochondria, neurofilaments and a moderate number of synaptic vesicles were also observed. However, some characteristics such as the membrane undercoating were not conspicuous in all the studied cells. Synaptic contacts were observed but with a lower frequency than in the prolongations. These synapses were normally established from an adjacent prolongation to the single identified axonal shaft of the ruffed cell and could be classified as symmetrical.

Due to the highly irregular contour of the protrusions, their profiles normally appeared isolated from the axonal shaft (Fig. 3). The ruff protrusions identified, despite their demonstrated axonal nature, exhibited a dendritic aspect. Thus, the typical characteristics of the IP such as fascicles of microtubules, clusters of ribosomes and, specially, the membrane undercoating, were lacking. In the interior of the protrusions, mitochondria, isolated cisternae of smooth endoplasmic reticulum — in aspect reminiscent of spine apparatus — and extremely abundant synaptic small clear vesicles were observed (Figs. 4, 5). Several dense-core vesicles were also observed. Additionally, symmetrical, asymmetrical and reciprocal synapse differentiations were found between these protrusions of the identified gold-toned neuron and unlabeled terminals (Figs. 4, 5). Synapses from the protrusion to the adjacent prolongation, presumably a granule cell according to its ultrastructural characteristics, could be classified as asymmetrical (Fig. 4B, 4C), the prolongation-to-protrusion synapses being symmetrical (Figs. 4D, 5C).
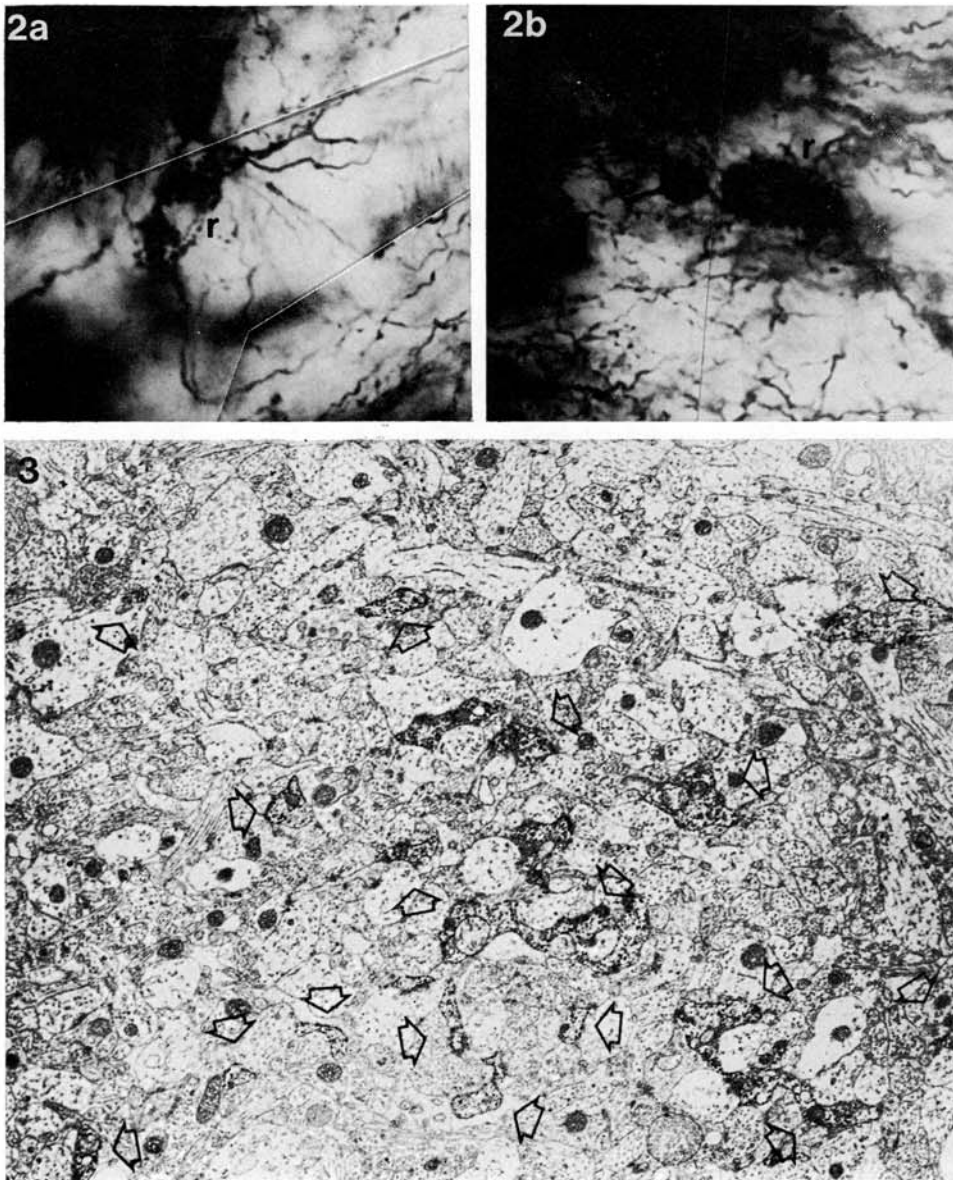
The majority of the terminals impinging on the IP

Fig. 2. Photomontages of two Golgi-impregnated ruffed cells which were subsequently sectioned for electron microscopy (r: ruff). × 380

Fig. 3. General view of the glomerular layer showing numerous protrusions of a single-identified ruffed cell axon. Profiles of the ruff (arrows) are clearly labeled by numerous gold-grains. × 4500

protrusions showed an intermediate size and were densely filled with small clear synaptic vesicles (Figs. 4C, 5A). These vesicles in the terminals had a smaller diameter than the small clear vesicles of the protrusions. One or two large dense-core vesicles and a mitochondrion could also occasionally be observed. These boutons established normally asymmetric contacts with the protrusion and, more rarely, with the axonic shaft in the ruff. Moreover, symmetric synapses from the protrusions to the unlabeled terminal, and reciprocal pairs (Fig. 4D), reminiscent of the synaptology found between the prolongations of granule cells and the dendrites of mitral cells, were also found. In some cases, the boutons contacting with the identified protrusions of the ruff also received an asymmetric synaptic contact from another termi-

nal with similar morphological characteristics, forming a serial synapse. The same terminal could also contact two different zones of a protrusion or, even, two protrusions of the same neuron.

In lower numbers, terminals with a similar size and with a higher number of dense-core vesicles and with a low number of small clear vesicles were observed to contact the identified ruff protrusions. Finally, other larger terminals showing small vesicles and with an "empty" appearance were also found to contact the ruff (Fig. 4D). These latter terminals occasionally received contacts from the most numerous group of typical boutons with small clear vesicles. In the serial sections, the same Golgi-impregnated protrusion could be observed to contact all three groups of terminals.
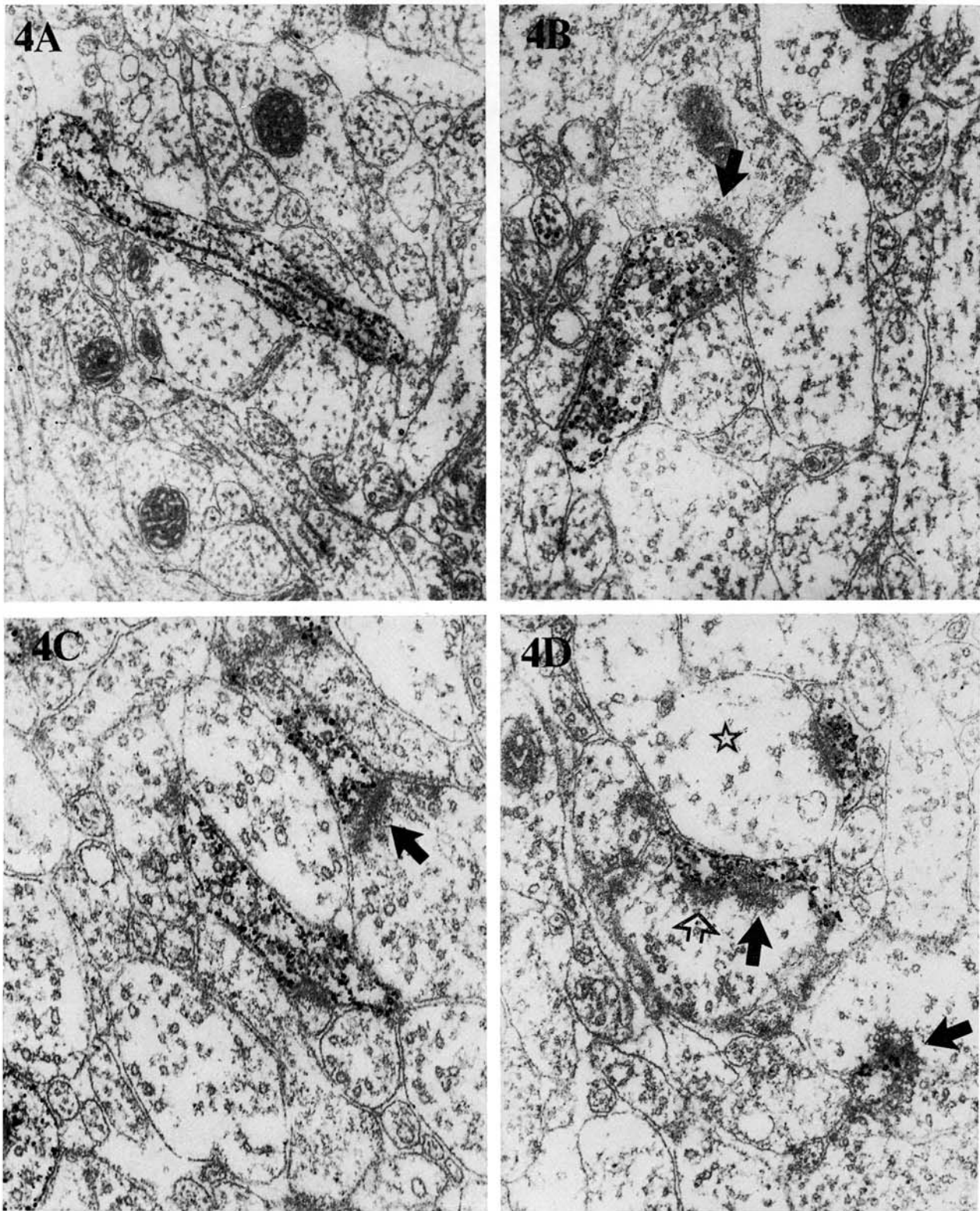
Fig. 4. Gold-toned profiles of a ruff. Note in all the cases the presence of numerous, round, clear synaptic vesicles. A: Part "3" of the initial segment of the axon. ×21 000. B—D: Symmetric and asymmetric synaptic contacts (open and close arrows) and pairs of synapses (Fig. D) were observed between the labeled axon initial segment and unidentified terminals with a reduced number of synaptic vesicles and organelles (star), presumably belonging to granule cells. ×29 000
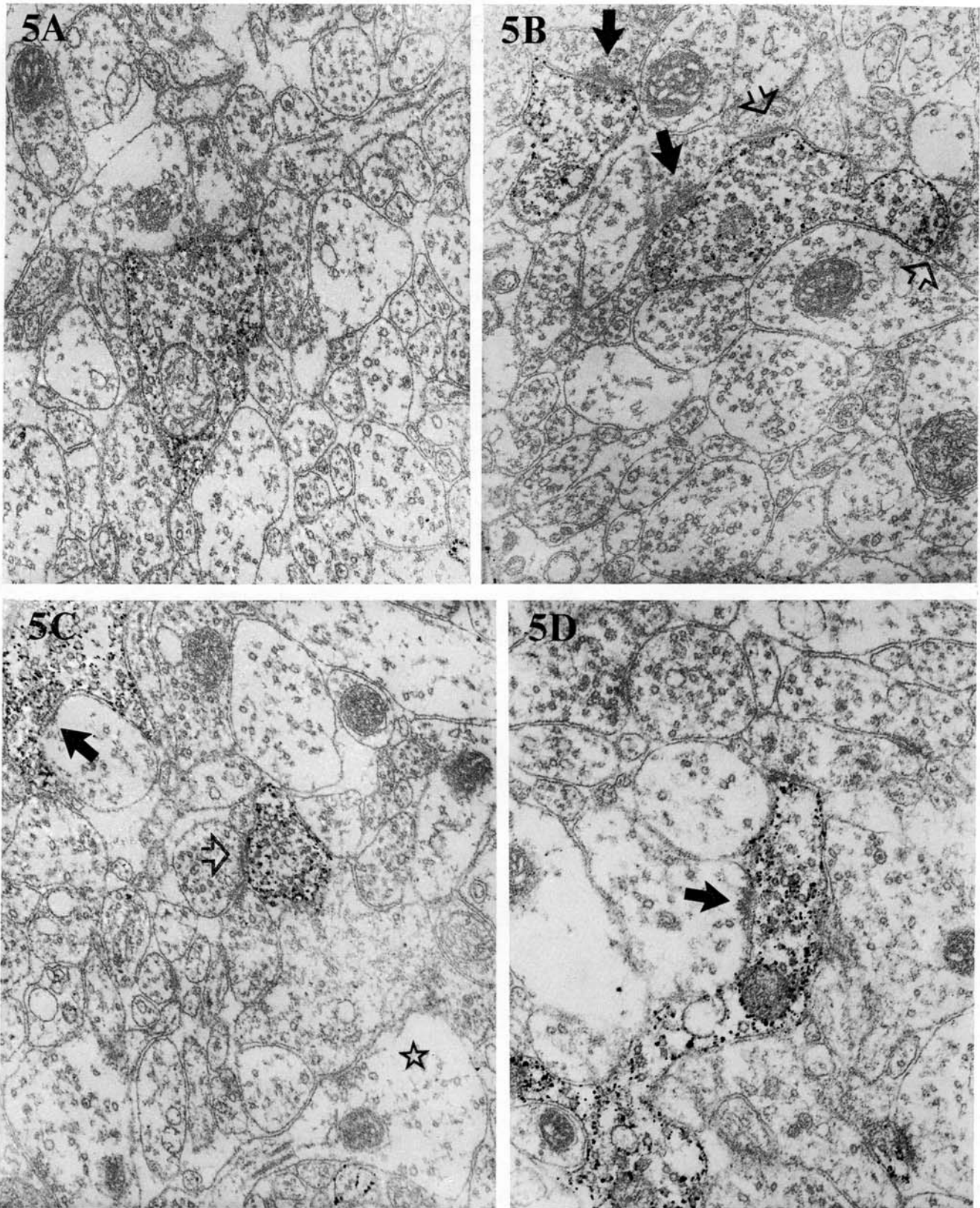
Fig. 5. Different profiles of identified ruff protrusions establishing synaptic contacts with unstained elements. Synapses from the protrusion to the adjacent prolongation, could be classified as asymmetrical, the prolongation-to-protrusion-synapses being symmetrical. × 29 000

Discussion

The light microscopic characteristics of the ruffed cell IP in *Hemichromis bimaculatus* are more similar than those observed in the cyprinoids *Barbus meridionalis*, *Carassius auratus*, *Carassius carassius*, *Chondrostoma polylepis*, *Cyprinus carpio* and *Tinca tinca*, the silurid *Parasilurus asotus*, and the salmonid *Salmo gairdneri* (KOSAKA and HAMA, 1979a, 1980, 1982–83; ALONSO et al., 1987) and are more different from the IP of the ruffed cells of *Conger myriaster* (KOSAKA and HAMA, 1980). Thus, in the former species part "0" is very short or practically inexistent and parts "1", "2" and "3" are easily identifiable in most cells. On the contrary, the ruff of *Conger myriaster* shows a long part "0" with a smooth contour and no protrusions, and in some cells of *P. asotus* only two different parts may be differentiated in the IP (KOSAKA and HAMA, 1980). Regarding the types of distribution of the dendrites in the ruffed cells, four different arrangements have been described (ALONSO et al., 1987). The dendritic arborization of the ruffed cells in *H. bimaculatus* coincides with previous observations on *Chondrostoma polylepis*, *Tinca tinca*, *Salmo gairdneri* and *Conger myriaster*. Thus, it is possible to assert the existence of a characteristic typology in the dendritic and axonic prolongations of the ruffed cells which is unrelated to the fact that the specimens belonged to a particular order of Teleosts (ALONSO et al., 1987).

The IP of the ruffed cells of *Hemichromis bimaculatus* shows the typical ultrastructural characteristics described in other neurons of higher vertebrates (PETERS et al., 1976) and also in the ruffed cells of other species of teleosts (KOSAKA, 1980; KOSAKA and HAMA, 1979a, 1980). The structure of the IP as postsynaptic region is well known (PALAY et al., 1968; JONES and POWELL, 1969; WILLEY, 1973; CONRADI and RONNEVI, 1977; KOSAKA and HAMA, 1979c, 1982–83). However, there is a reduced group of examples of neurons showing an IP as a presynaptic zone located in different brain regions: the monkey dorsal lateral geniculate nucleus (HAMORI et al., 1978), the cat cerebellar cortex (HAMORI, 1981), and the cat, rat, and fish olfactory bulbs (PINCHING and POWELL, 1971; WILLEY, 1973; KOSAKA and HAMA, 1979a, 1982, 1982–83). Peculiar of the ruffed cells, both *Hemichromis bimaculatus* and other species previously studied (KOSAKA and HAMA 1979a, 1980, 1982–83), is the presence of numerous presynaptic and postsynaptic sites located on the IP protrusions. In the olfactory bulb, presynaptic IPs are observed in different cell types such as the mitral cells of mammals (WILLEY, 1973) and teleosts (KOSAKA and HAMA, 1982), the middle tufted cells (PINCHING and POWELL, 1971) and ruffed cells

(KOSAKA and HAMA, 1979a, 1980). It is important to note that these cells include practically all the second order neurons — projecting to higher centers — present in the olfactory bulb. In the case of mitral and tufted cells, it seems that after a high convergence of olfactory inputs from the receptor cells upon a single neuron a strong system of inhibition located on the IP of these cells may be useful. However, this explanation is not valid for the ruffed cells since this neuronal type receives a very low number of synapses on the dendritic fields.

The protrusions of the ruff are clearly different in their structure and synaptic organization from other axonal specializations such as collaterals, bulges and spinous projections, small side branches, axonic spines and small protuberances. Our observations in *Hemichromis bimaculatus* also confirm that their connection pattern is very complex. It must be emphasized that the synaptology of the ruff resembles the contacts between the mitral cells and the granule cells, as they are observed in the teleostean olfactory bulb; and those between mitral cells and granule cells, and tufted cells and granule cells in the mammalian olfactory bulb. Teleostean mitral cells are, in several aspects, clearly different from mammalian mitral cells (ALONSO et al., 1988; FUJITA et al., 1988). The mitral cells of the olfactory bulb of teleosts also show a high number of reciprocal synapses and presynaptic and postsynaptic differentiations in the initial portion of the axon (KOSAKA and HAMA 1982; OKA, 1983). KOSAKA and HAMA (1982) have reported that in random sections synapses are more commonly encountered on the axon hillock and the initial segment than on the soma and the dendritic stem. However, the number of synaptic inputs on the dendritic arborizations of the mitral cells is very high.

Elaborated systems of protrusions are not unique in the teleost olfactory bulb. Thus, KOSAKA and HAMA (1979b) described that the IP of the ruffed cell resembles some neuronal processes of the invertebrates as described by BULLOCK and HORRIDGE (1965). More recently, LOPEZ-MASCARAQUE et al. (1986) have shown in the olfactory bulb of *Erinaceus europaeus* a similar disposition around a dendritic process of a granule cell. Although these authors have not carried out a study on these neurons using electron microscopy, their structure, location and, probably, connections are different from those of the teleostean ruffed cell. Moreover, although these protrusions are also supposed to be "a device engaged in a powerful control of local interactions", they are disposed on a dendritic prolongation and not on the IP.

The three group of terminals previously described:

intermediate-sized and numerous small clear vesicles, intermediate-sized with a group of dense-core vesicles and larger terminals showing small clear vesicles and having an "empty" appearance may be morphological variants belonging to the same neuronal type or belonging to different cell types. In this sense, "empty" profiles have been described to belong to perinest cells (Kosaka and Hama, 1982—83). In any case, the majority of the contacts impinging on the protrusions of the ruff, with intermediate size and numerous small clear vesicles, can be identified as arising from granule cells. The granule cells — the most important interneuron of the olfactory bulb in all vertebrates — show a great morphological diversity in the teleostean olfactory bulb (Alonso et al., 1986). According to our results and previous descriptions, they are also involved in new neuronal circuitries not present in mammals. We have observed in our material that a bouton establishing a synapse with a single-identified protrusion contacts in the same or another plane with another unidentified terminal in a reciprocal pair. This latter prolongation could be, according to its ultrastructural characteristics, a protrusion from a second unlabeled ruffed cell, or a dendritic process from a mitral cell, indicating, in any case, a high degree of complexity of the synaptic interactions in the glomerular layer between different cell types hitherto not previously described in any other vertebrate.

The ruffed cell has previously been described in the order Cypriniformes, order Salmoniformes, and order Anguilliformes (Kosaka and Hama, 1979a, 1980; Alonso et al., 1987). Our study in *Hemichromis bimaculatus* extends these observations to a new order and strongly supports the idea that the ruffed cell is a characteristic cellular type presumably common to all teleosts (Kosaka and Hama, 1980; Alonso et al., 1987). In this sense, it is interesting that although Fujita et al. (1988) have not found ruffed cells in the olfactory bulb of *Cyprinus carpio* using intracellular injections, we have observed such cells in the same species using different variants of the Golgi technique (Alonso et al., 1987). This discrepance may be due to the fact that ruffed cells were observed in the outer region of the glomerular layer and more superficial injections than those done by Fujita et al. (1988) may be necessary to demonstrate such neurons. It would be important to continue these experiments since the intracellular injections can fill the axons and their collaterals for a long distance, demonstrating the projection regions of the ruffed cells and their similitudes and differences with the target fields of the mitral cells. This might clarify the presently unknown function of the ruffed cells.

No explanations have been proposed for the possible meaning of the elaborate structure of the ruff and the particular disposition of synaptic inputs in the ruffed cells. Thus, these neurons exhibit a large increase in surface area in the axonal IP, where an extremely high number of synapses is observed; on the other hand, as previously mentioned, the dendritic branches are very thin and the synapses in this region are comparatively scarce. This could in dicate a fast response system where a single portion of a neuron, the ruff, interacts directly and indirectly with numerous neurons, granule cells and perinest cells respectively (Kosaka and Hama, 1982—83), modulating the information relay produced between the first and second elements of the olfactory pathway, the olfactory fiber and the mitral and ruffed cells axons. Accordingly, the concept of the synapses on the IP as a system of output control, where a reduced number of presumably inhibitory synapses can block the spike originated as a result of a much higher number of dendritic inputs, at least for the ruffed cell, should be reconsidered.

Acknowledgements

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