

Effects of chronic low-level copper exposure on ultrastructure of the olfactory system in rainbow trout (*Oncorhynchus mykiss*)

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Summary. This study investigated the effects of a chronic exposure to a low level of copper on cell populations of the olfactory system in yearling rainbow trout. Fish were sacrificed after 15, 30 and 60 days of copper exposure. Transmission electron microscopy was used to describe the sequence of subcellular changes occurring in three tissues, the sensory epithelium, the olfactory nerve and the olfactory bulb. Data show that a 15-day exposure to 20 µg/l of copper causes specific degeneration of all mature receptor cells as well as numerous immature neurons. Moreover, degenerating receptor cells exhibited morphological features of a cell death by apoptosis. After 30 days, and more specifically after 60 days of exposure, numerous clusters of cells were observed in the basal region of the epithelium, suggesting a great mitotic activity in this area. In parallel, an increased number of maturing receptor cells and goblet cells were observed, but no fully mature neurons were noted even after 60 days of exposure. In both the olfactory nerve and the olfactory bulb, the number of degenerating axons and terminals, which was high at 15 days, decreased with time and some process of glomerular reinnervation was detected after 60 days. A reactive hypertrophy of supporting, ensheathing and astrocytic cells was also observed in exposed fish, which demonstrates that these cell types are actively involved in the process of tissue scarring. Even though some signs of neuronal regeneration were reported during the time-course of exposure, indicating some fish acclimation, results raise the question of the olfactory function during such environmental stress.

Key words: Olfaction, Ultrastructure, Toxicology, Copper, Fish

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Introduction

Oligoelements, like copper, are essential micro-nutrients for living organisms. In aquatic biota, copper may become toxic if it exceeds the standard nontoxic value which is established at 5-15 µg/l (Birge and Black, 1979). The fish olfactory organ is particularly vulnerable to toxicants since its receptor cells are directly in contact with the external environment. The olfactory receptor cells are bipolar neurons with a ciliated or microvillar dendrite emerging at the epithelial surface and with an axon ending in the olfactory bulb, which is the first relay for information processing in the central nervous system (SNC).

Histopathological studies have highlighted the action of copper in fish olfactory organ showing that short-term exposures to high (Gardner and LaRoche, 1973; Gardner, 1975; Brown et al., 1982; Hara et al., 1983) or moderate (Klima and Applehans, 1990) copper concentrations impair the olfactory organ and induce degeneration of receptor cells. Likewise, long lasting exposures to a copper level as low as 20 µg/l in rainbow trout, from eyed stage or from hatching, also induce lesions in the olfactory lamellae, but with a much longer delay (Saucier et al., 1991). The latter experimental design has the advantage of showing sequential action of the toxicant upon the olfactory organ, but observations being performed by light microscopy it is not unlikely that some subcellular changes could not be detected in the different cell types of the epithelium. Recent data of Moran et al. (1992) seem in accord with this assumption since about half of the olfactory receptor cells had degenerated in brown trout exposed to 18 µg/l of copper for one day.

The purpose of the present study is to investigate by transmission electron microscopy, the ultrastructural changes taking place in the olfactory system in response to a chronic exposure to a low copper concentration in adult rainbow trout (*Oncorhynchus mykiss*). We have been interested in following the sequence of cell changes

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occurring during an exposure period as long as 2 months. The analysis was performed at the level of the sensory epithelium which comprises four morphological distinct cell types: olfactory receptor cells; ciliated non-sensory cells; supporting cells; and basal cells (Bertmar, 1972; Zeiske and Melinkat, 1976; Theisen et al., 1980; Zielinski and Hara, 1988; Moran et al., 1992). To obtain an overview of cell alterations occurring in the olfactory system, the olfactory nerve and the more central olfactory bulb were also studied. Data show that copper selectively impairs the olfactory receptor cells, whereas the other cell types remain relatively intact.

Materials and methods

Yearling rainbow trout (*Oncorhynchus mykiss*) were obtained from the Aquarium and Centre Marin of Shippagan (N.B.), Canada. Fish were kept in 500 l circular fiberglass tanks with a flow-through of 5 l/min. Two experimental groups were considered: (1) an exposed group was placed in well water in which a calculated quantity of CuSO_4 was perfused into the incoming water line of the tank with a peristaltic pump in order to maintain an average copper concentration of 20 $\mu\text{g/l}$; and (2) a control group was left in well water. The main water parameters were as follows: temperature, 4.6-5.8 °C; pH, 6.53-6.96; dissolved oxygen, 10.5-11.8 mg/l; total hardness, 62-64 ppm as CaCO_3 . Temperature was checked daily and the other parameters weekly. An average copper concentration of $20 \pm 1.4 \mu\text{g/l}$ as determined by atomic absorption spectrometry was maintained in the tank of the exposed group. Fish were kept under illumination from 8 h until 18 h and were fed daily with trout grower dry pellets.

On days 15, 30 and 60 of copper exposure, 4 specimens of the exposed group were sacrificed. Four fish of the control group were also sacrificed at day 30. After decapitation, rosettes were quickly removed from the olfactory cavities and put into a mixture of 1% glutaraldehyde and 0.5% paraformaldehyde in monosodium dipotassium phosphate buffer 0.1M, pH 7.4, for 2 h. Olfactory nerves and bulbs were fixed *in situ* for 1 h and then dissected and put in fresh fixative for 2 h. After several rinsings in 0.16M phosphate buffer for 2 h, samples were postfixed with 2% osmium tetroxide buffered with 0.1M phosphate buffer for 1 h. Dehydration was performed in a graded ethanol series and then tissues were embedded in Epon 812. Semi-thin and ultra-thin transverse sections of olfactory lamellae were made and frontal sections were performed from nerve and bulb samples. Semi-thin sections were stained with a mixture of azurII-toluidine blue. Ultra-thin sections were collected on copper slot grids coated with formvar and stained with a methanolic solution (7%) of uranyl acetate followed by lead citrate.

Results

15 days of copper exposure

As shown by light microscopy (Fig. 1), the olfactory epithelium of exposed fish presented no injury and appeared well organized, but it exhibited a rather uniform translucent staining compared to controls. This was mainly due to the fact that the typical mature olfactory receptor cells, characterized by a dark staining and by a prominent dendritic knob (Fig. 1a), were absent in exposed fish (Fig. 1b). Only supporting and ciliated

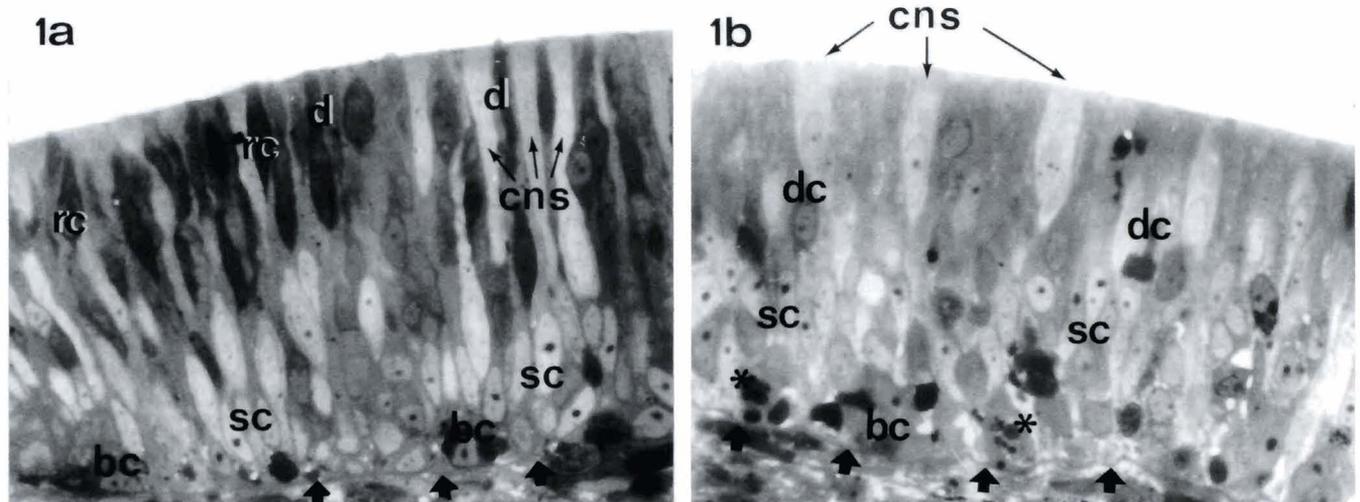


Fig. 1. Light microphotograph of a cross-sectioned lamella through the sensory epithelium of control fish (a) and of fish exposed to copper for 15 days (b). a: In control fish, the dark-stained receptor cells (rc) have a large dendrite (d) which emerges in the luminal cavity. The nuclei of the ciliated non-sensory cells (cns) are mainly located in the superficial half of the epithelium while those of the supporting cells (sc) are located in the basal region. b: In exposed fish, only ciliated non-sensory cells, supporting cells and few dark cells (dc) of irregular shape can be observed. Asterisks show degenerating cells. As for the next figures, basal lamina is indicated by large solid arrows. bc = basal cells. a, b: x 1,100

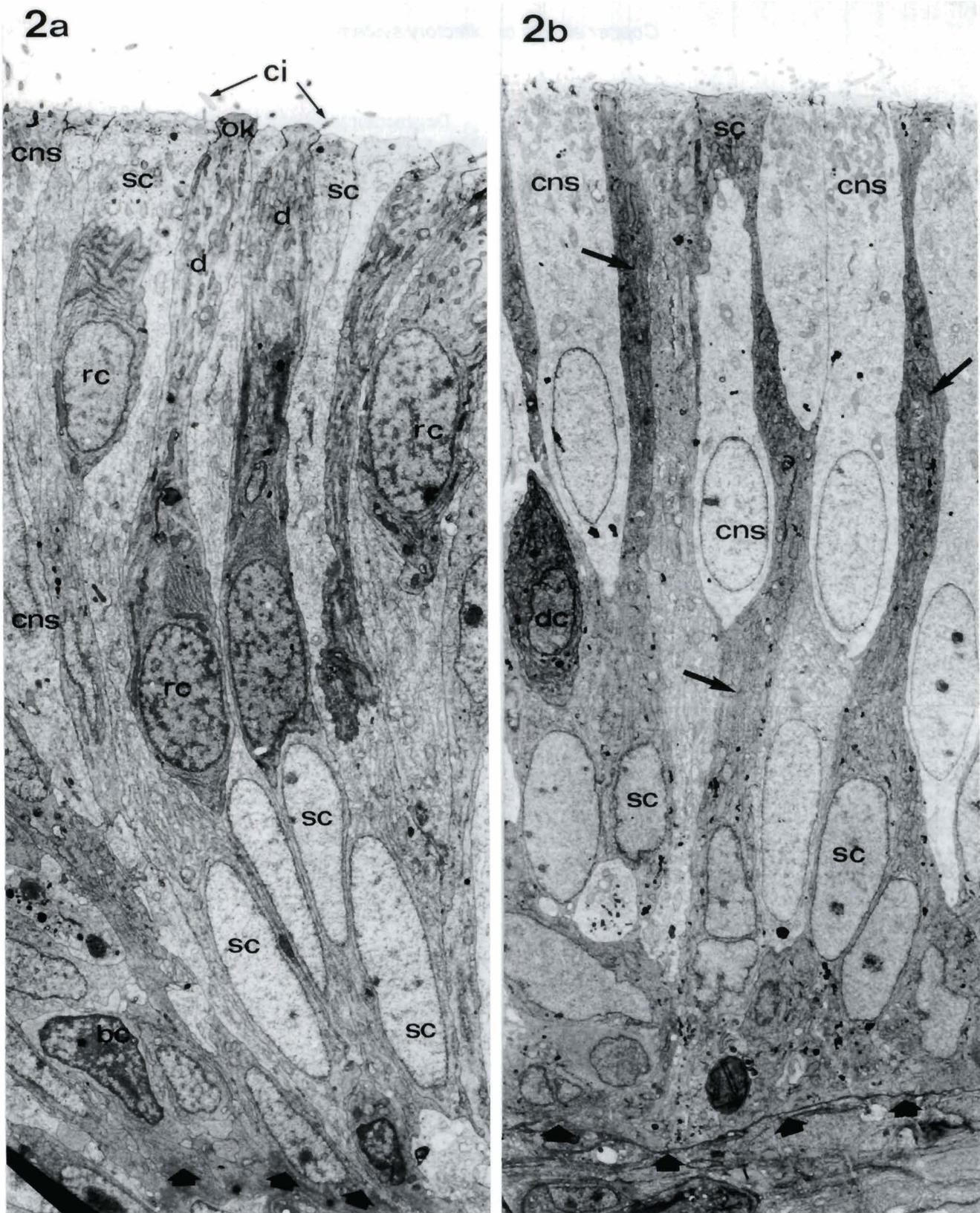


Fig. 2. Electron micrographs show the distribution of the different cell types forming the sensory epithelium in control (a) and in fish exposed to copper for 15 days (b). a: In control fish, the receptor cells (rc) are easily recognizable with their electron-dense cytoplasm and nucleus. Their large dendrite (d) reaches the free surface and expands into an olfactory knob (ok) which is provided with cilia (ci) or microvilli in fully mature neurons. The cytoplasm and nucleus of the supporting cells (sc) and ciliated non-sensory cells (cns) appear electron-lucent. The apical surface of the ciliated non-sensory cells bears small microvilli and numerous long cilia arising from basal feet which are associated with striated rootlets. bc = basal cell. x 3,600. b: In exposed fish, only supporting and ciliated non-sensory cells could be easily recognized. Supporting cells usually present an electron-dense and hypertrophied cytoplasm (arrows). One dark cell (dc) exhibiting typical features of maturing receptor cell is also observed. x 2,700

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non-sensory cells as well as few scattered goblet cells could be recognized in this experimental group (Fig. 1b). Few irregularly-shaped dark cells and degenerating cellular elements were also reported.

Electron microscopy observations (Fig. 2) confirm the absence of mature olfactory receptor cells in the exposed group. Compared to controls (Fig. 2a), exposed fish exhibited supporting cells whose cytoplasm seemed enlarged with many mitochondria and rough endoplasmic reticulum cisternae that could be dilated (Fig. 2b). Thus, supporting cells looked hypertrophied, some cells having an electron-dense appearance. The cell expansions extended radially surrounding adjacent ciliated non-sensory cells and they presented a horizontal extension of their apical profile (Fig. 2b). Two additional organelles were observed in supporting cells of exposed fish. Porous and stacked parallel membranes, named annulate lamellae, were seen near the nucleus of many cells where they often appeared in continuity with the rough endoplasmic reticulum and mitochondria (Fig. 6, insert). Numerous supporting cells also contained lipid droplets, the largest ones being found in the lowest part of the epithelium where they were closely associated with the rough endoplasmic reticulum (Fig. 3c). The ciliated non-sensory cells presented no evident ultrastructural alteration except an increased number of aberrant expansions at the apical surface of the epithelium and the presence of some lipid droplets.

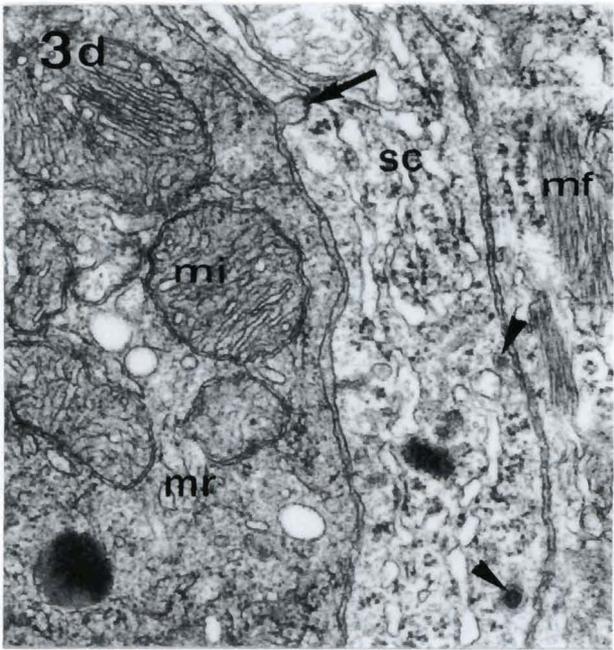
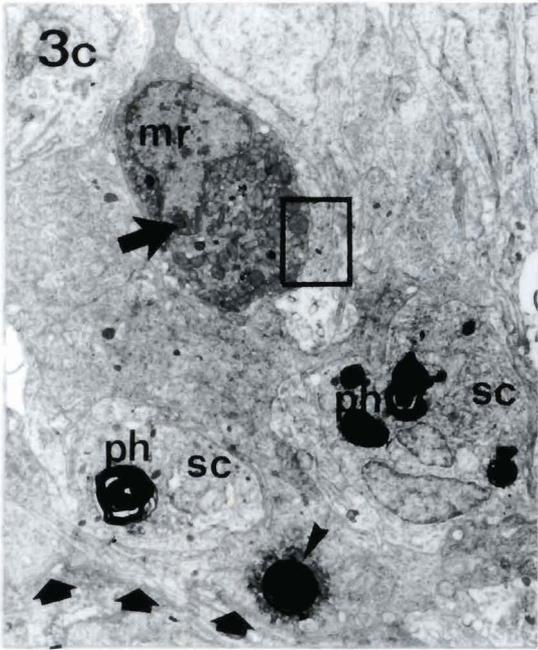
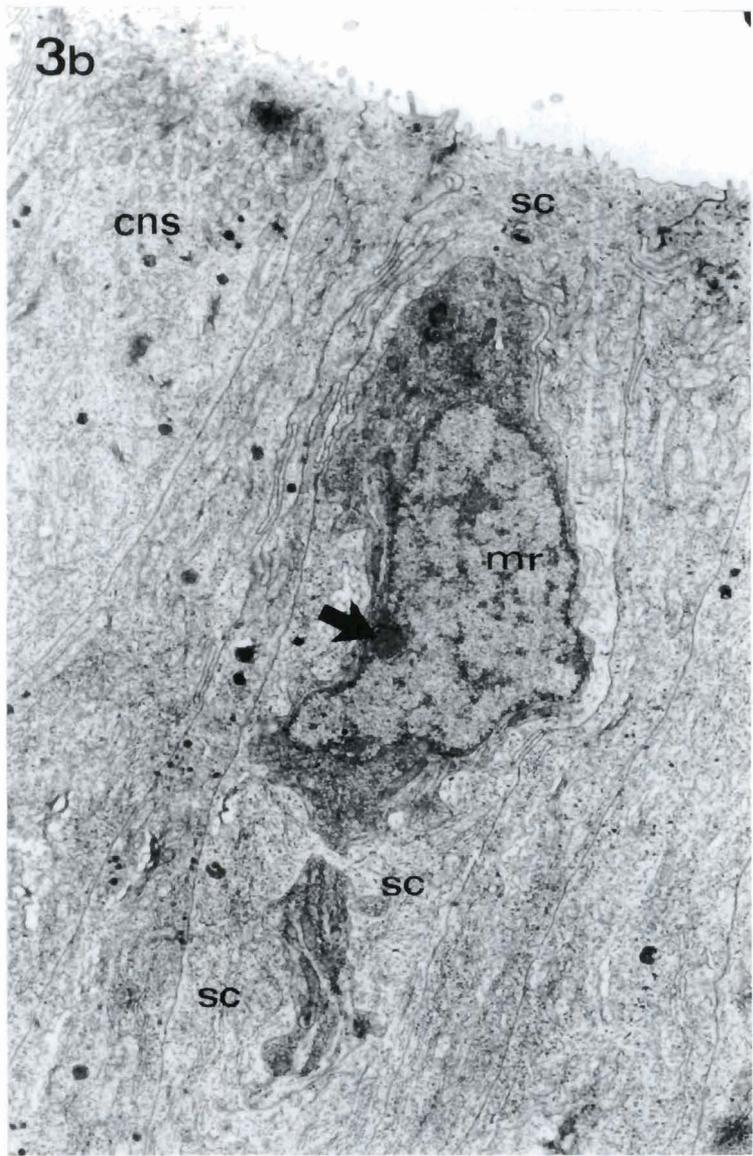
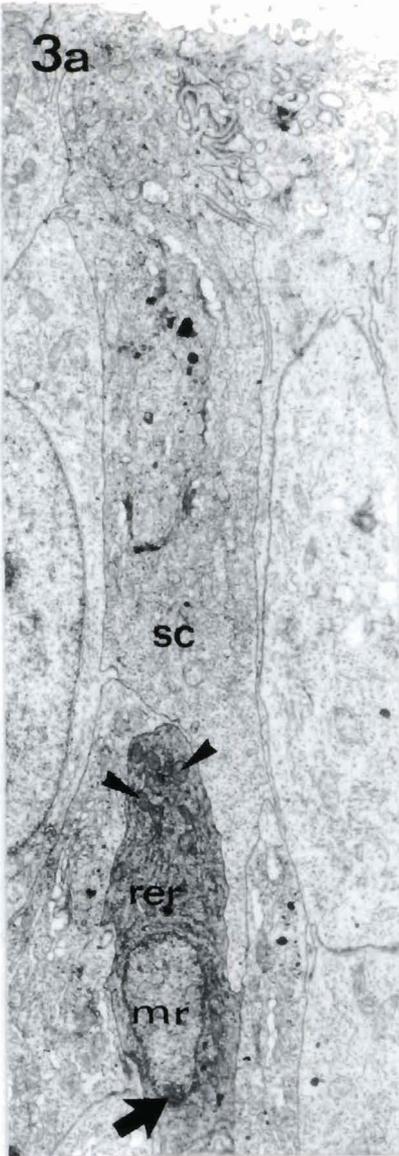
Some electron-dense cells showing a dark staining in light microscopy were also noted in the olfactory epithelium of exposed fish (Fig. 2b). The dark cells located in the apical half of the sensory epithelium were mainly characterized by a large supranuclear cytoplasmic region containing electron-dense mitochondria, free ribosomes and rough endoplasmic reticulum cisternae organized in orderly stacked arrays (Fig. 3a). Their nucleus exhibited a large peripheral condensation of heterochromatin with only one small eccentric nucleolus (Figs. 3a,b). These dark cells presented morphological features of maturing receptor cells. Their distal processes were never seen emerging in the luminal cavity from which they were always separated by processes of supporting cells that surrounded the neuron perikaryon (Fig. 3b). Dark cells located in the basal part of the epithelium presented a large polarized infranuclear cytoplasm containing numerous electron-dense mitochondria (Fig. 3c). As shown in Figure 3d, some coated vesicles were seen in areas where the plasma membranes of both maturing neurons and supporting cells were in close apposition.

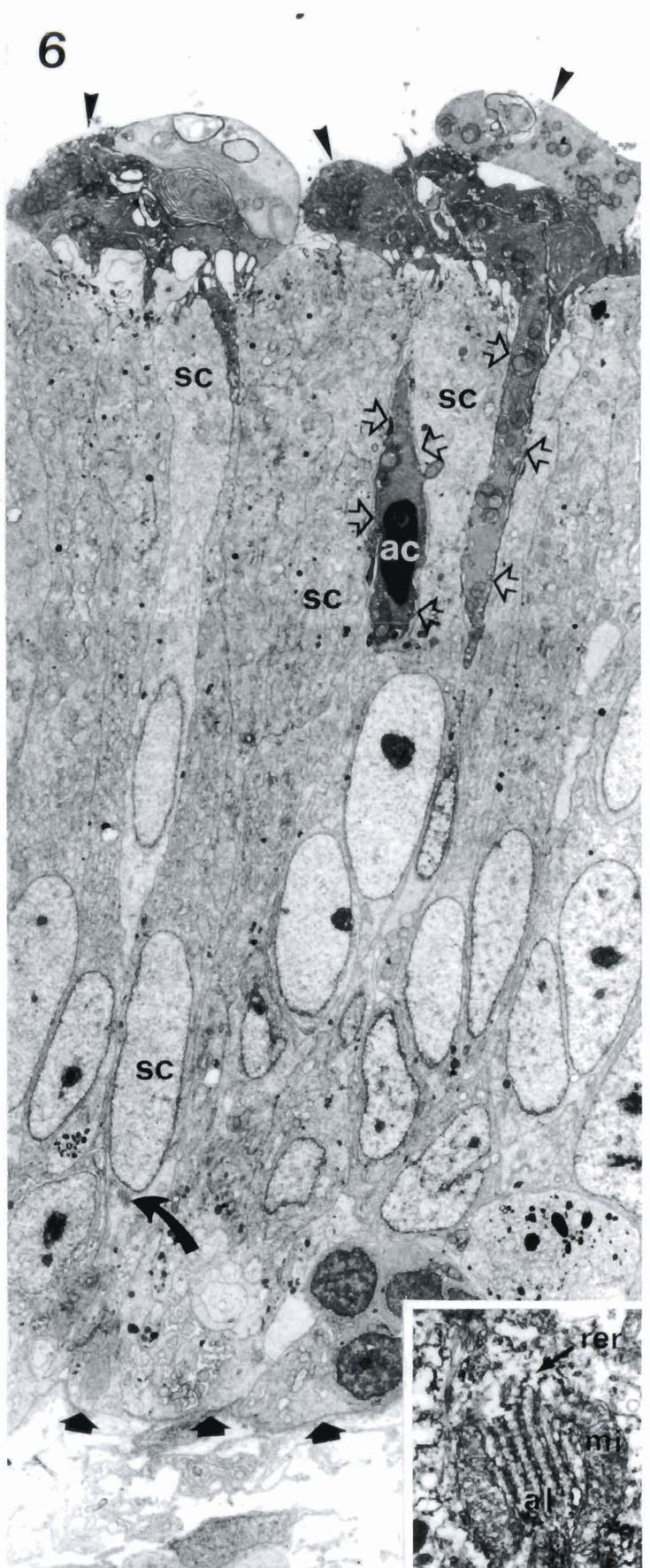
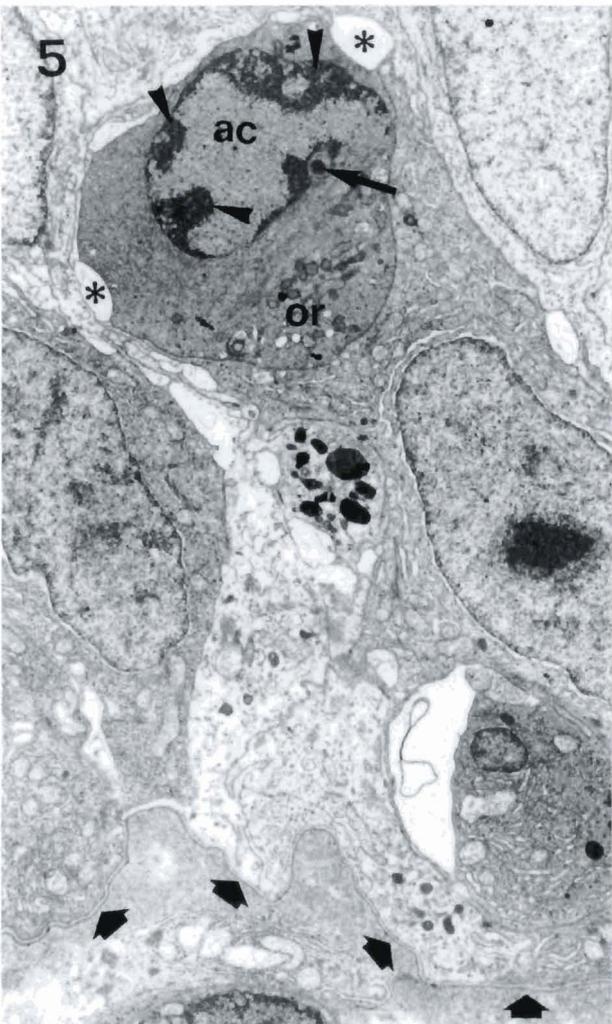
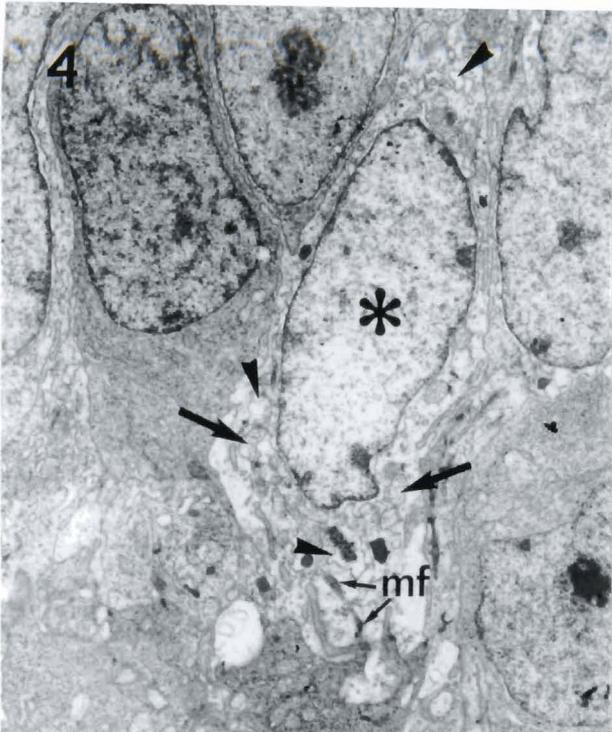
Degenerating cellular elements were also present in the sensory epithelium of exposed fish. Two morphological types of cellular changes leading to cell death could be distinguished. In few cases, supporting and ciliated non-sensory cells exhibited ultrastructural features of necrosis characterized by a cell swelling and the presence of dilated and broken organelles (Fig. 4). A few necrotic cells were also found in controls. On the other hand, dying cells could exhibit the morphological features of a cell death by apoptosis. In these cases, as shown in Figure 5, the chromatin presented a typical pattern of intensively osmiophilic condensation and filled a large part of the nucleus. The nucleolus was enlarged and looked like a less intensively osmiophilic fibrillar body well demarcated from the peripheral condensed chromatin by a distinct margin. Organelles in the cytoplasm were packed together but they remained structurally intact until the late stages of cell degeneration. Apoptotic cells located in the upper part of the epithelium appeared to be extruded in the cavity lumen. These cells, which exhibited a typical cytoplasmic protrusion with a mushroom-like shape pouring out at the epithelial free surface (Fig. 6), were probably degenerated mature receptor cells. These electron-dense cells were confined between supporting cells with which they were in close apposition, but no junctional specialization was seen between both cellular types (Fig. 6). On the other hand, apoptotic cells located in the lower part of the sensory epithelium were mainly eliminated by phagocytosis. These cells, which were characterized by their ovoid shape (Fig. 5), seemed to be immature receptor cells. They were firstly broken into a series of membrane bounded fragments containing an assortment of organelles and were then phagocytosed by neighbouring cells (Fig. 7). Vacant sites were mainly occupied by large translucent intercellular spaces containing membrane remnants.

In light and electron microscopy observations, no mitotic activity was detected in basal cells directly in contact with the basal lamina. One could observe a few cell divisions in the area just above the basal cells (Fig. 8). Some supporting cell mitoses were also noted in the upper part of the sensory epithelium.

In the olfactory nerve of exposed fish, profiles of degenerating axons with typical electron-dense axoplasm were intermingled with those of intact axons (Fig. 9a). The number of intact axons was greatly reduced and bundles of packed axons appeared smaller in size (Fig. 9b). Ensheathing cells surrounding axon bundles often exhibited swollen processes of tongue-like

Fig. 3. Examples of maturing receptor cells (mr) observed in the sensory epithelium of 15-day exposed fish. In a, the maturing receptor cell exhibits a developed distal process containing electron-dense mitochondria (arrowheads) and stacked rough endoplasmic reticulum cisternae (rer). In b, the maturing neuron which is located near the epithelium surface, appears completely surrounded by supporting cell (sc) processes. In c, the maturing receptor cell which is located in the deep part of the epithelium, exhibits a well developed infranuclear cytoplasm filled with numerous mitochondria. One can note the presence of a lipid droplet (arrowhead) and large phagosomes (ph) in the cytoplasm of the surrounding supporting cells. Large arrows in a,b and c point to the small eccentric nucleolus characterizing the maturing neuron nucleus. a: x 4,600; b: x 6,100; c: x 4,000. In d, detail showing the close apposition between the maturing receptor cell and a supporting cell. Arrow indicates a coated vesicle from supporting cell and arrowheads show dense-cored vesicles. x 28,000. cns = ciliated non-sensory cell, mf = microfilaments of supporting cell, mi = mitochondria





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Figs. 4 - 6. Electron micrographs showing cell death by necrosis and apoptosis in the sensory epithelium of 15-day exposed fish.

In **Fig. 4**, a supporting cell (asterisk) exhibits signs of early necrosis. Arrows point to swollen mitochondria and arrowheads to swollen endoplasmic reticulum. mf = microfilaments. x 5,900.

Fig. 5. Presents an example of apoptotic cell (ac). Arrowheads point to the large compact granular masses of heterochromatin abutting on the nuclear membrane. The nucleolus (arrow) is separated from condensed chromatin by an electron-lucent margin. Cytoplasmic organelles (or) are compacted together. Asterisks indicate the zones where the apoptotic cell loses contact with neighbouring cells. x 6,200

Fig. 6. Shows apoptotic receptor cells (ac) which are extruded in the olfactory organ lumen. Apoptotic neurons stay in close apposition (open arrows) with neighbouring supporting cells (sc). Arrowheads point to the cytoplasmic protusions containing compacted electron-dense mitochondria and long endoplasmic cisternae that appear unaltered. Incurved arrow shows annulate lamellae in the cytoplasm of a supporting cell. x 3,000. Insert: detail of annulate lamellae (al). x 24,000

shape (Fig. 9b). These processes that may be connected by desmosomes, contained inclusions whose shape could vary from multivesicular bodies to typical phagosomes. Large lucent vacuoles and lipid droplets were often associated with these phagosomes (Fig. 9a).

At the level of the olfactory bulb, light microscopy observations showed that in comparison to the control group (Fig. 10a), the glomerular layer of exposed fish

appeared rather disorganized (Fig. 10b) and was filled with granular material. Neither did the glomerular neuropiles appear well recognizable. The corresponding ultrastructural section (Fig. 10c) shows that granular material was in fact phagosomes surrounded by large, clear glial profiles. Inside these phagosomes, degenerating terminals of olfactory axons were still recognizable with their mitochondria and some typical

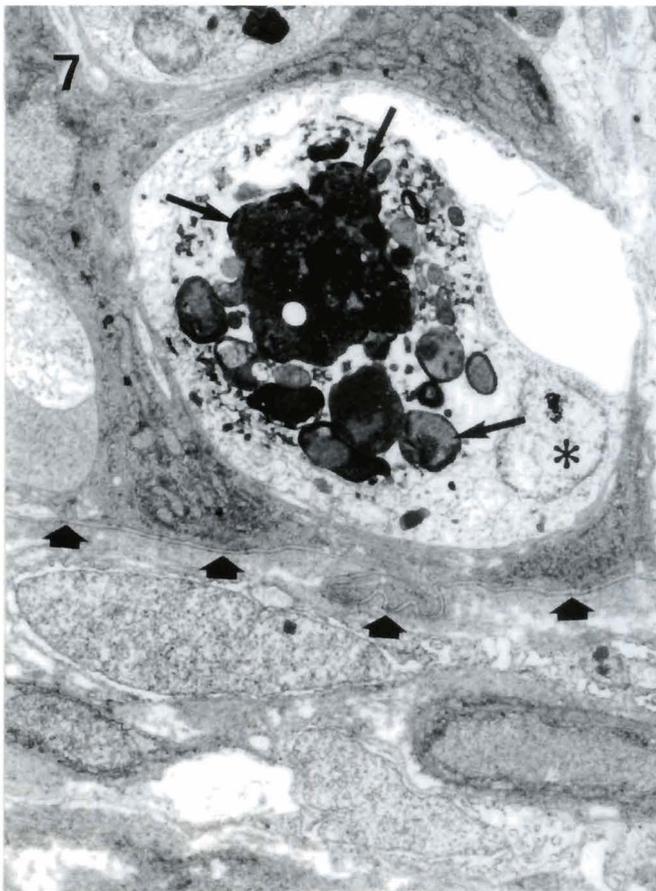


Fig. 7. Electron micrographs showing in the sensory epithelium of 15-day exposed fish apoptotic bodies (arrow) phagocytosed by a neighbouring cell (asterisk). x 6,100

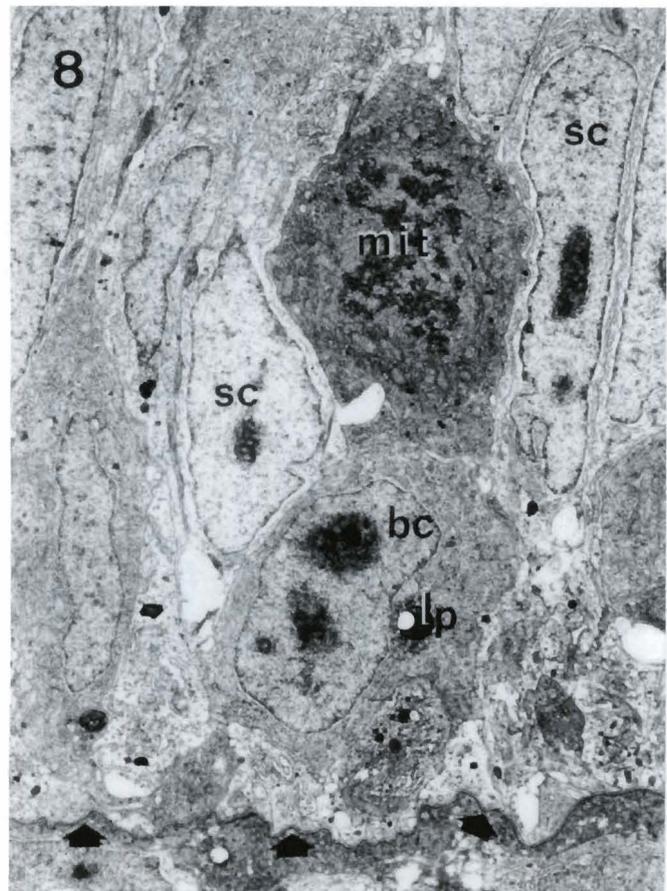


Fig. 8. Mitotic figure (mit) of a cell which is located just above a basal cell (bc) in 15-day exposed fish. sc = supporting cell, lp = lipid droplet. x 4,000

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synaptic vesicles. As shown in Figure 10c, some intact olfactory terminals still established asymmetrical synaptic contacts. Two types of clear enlarged profiles could be distinguished in the glomerular neuropil. The first refers to the astrocytic processes which filled large areas of the neuropil. These were easily recognizable by their electron-lucent appearance, the presence of phagosomes and lipid droplets (Fig. 10c). Such glial hypertrophy was also observed in vascular end-feet of astrocytes, and in glia limitans which presented large swollen and sometimes broken profiles. The second profile corresponds to the dendritic elements of the second order neurons which made synaptic contacts with olfactory axon terminals. Some contained mitochondria, scattered smooth endoplasmic reticulum, vesicles and a few microtubules. The others had no mitochondria nor

microtubule, but presented a diffuse network of microfilaments which is a typical feature of the dendritic growth cones (Fig. 10c).

30 and 60 days of copper exposure

After 30 days of exposure, no more degenerating cellular elements was detected in the sensory epithelium. Supporting and ciliated non-sensory cells exhibited roughly the same features as those seen in controls except that annulate lamellae were occasionally observed in supporting cells at day 30. One typical event related to the time-course of exposure was the presence of clusters of cells in the basal region of the epithelium, clusters whose number increased noticeably between the 30th and the 60th day of exposure. In light microscopy,

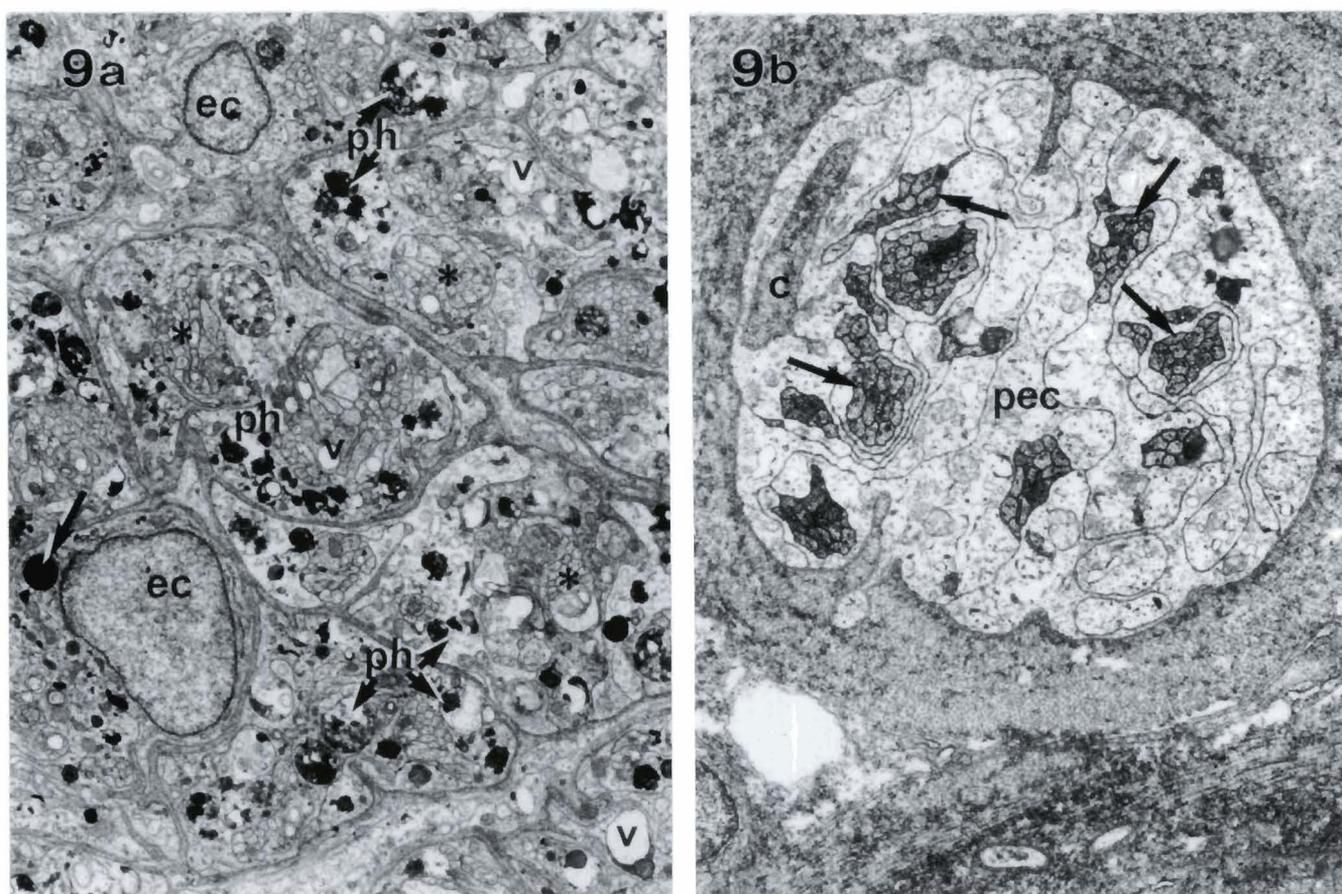
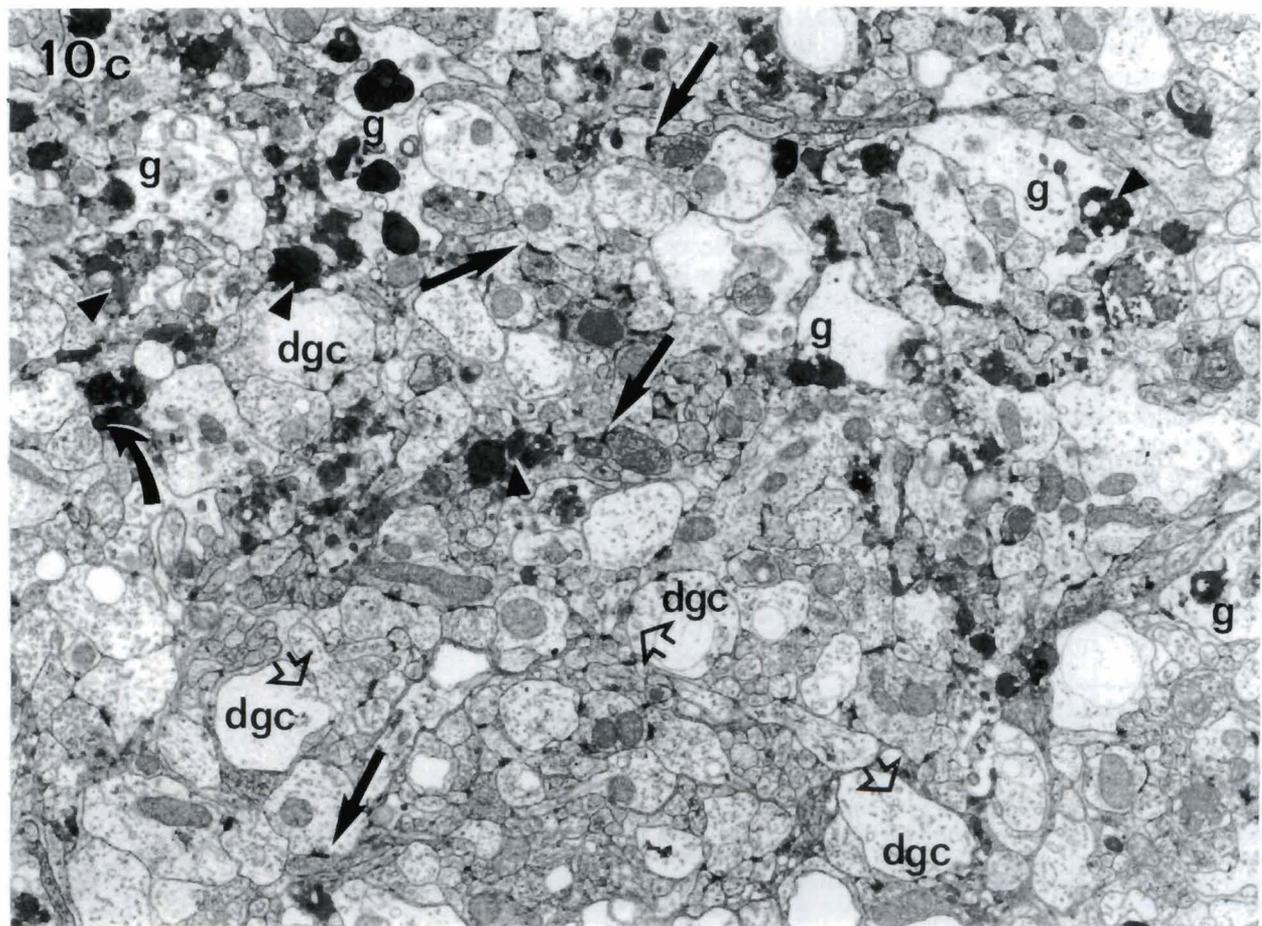
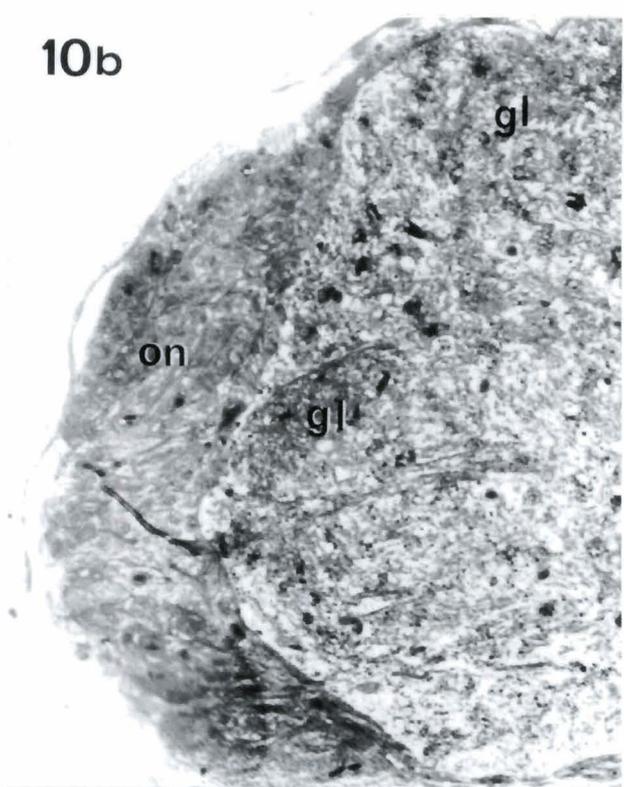
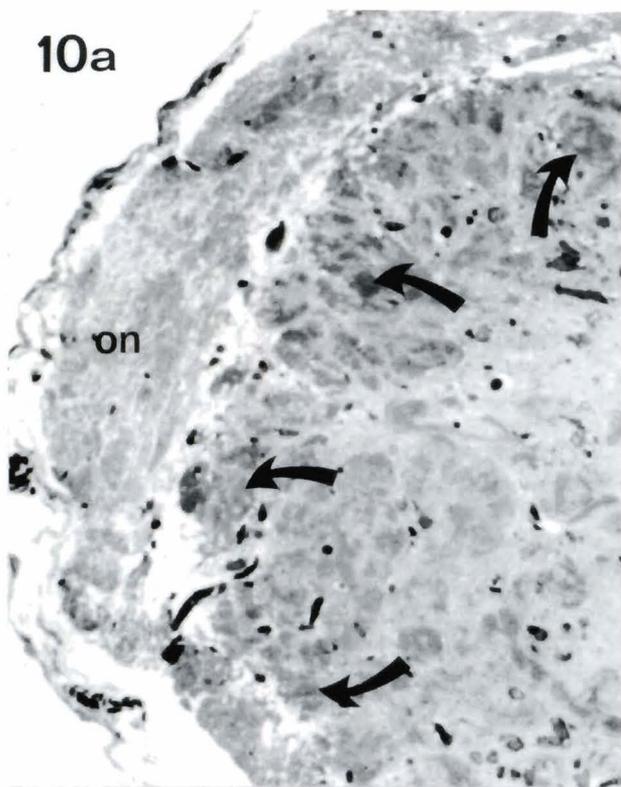


Fig. 9. Electron micrographs of the olfactory nerve in 15-day exposed fish. In a, many olfactory axons appear degenerated. Numerous phagosomes (ph) and large translucent vacuoles (v) are present in ensheathing cells (ec). Some intact axons are grouped in small bundles (asterisks). Arrow points to a lipid droplet. x 5,000. In b, some processes of ensheathing cell (pec) appear hypertrophied and surround small bundles of intact axons (arrows). One can observe a pocket of extracellular space with collagen fibres (c) partially enclosed by these processes. x 11,000

Fig. 10. a, b. Light microphotographs of the olfactory bulb in a control (a) and a 15-day exposed fish (b). In a, incurved arrows indicate glomerular neuropils. In b, the glomerular layer (gl) is filled with small granular material. on = olfactory nerve. a, b: x 300. In c. Electron micrograph of the bulbar glomerular layer in 15-day exposed fish. Many olfactory axon terminals (arrowheads) appear degenerated and are wrapped in hypertrophied clear astrocytic profiles (g). Arrows point to asymmetrical synapses. Dendritic growth cones (dgc) of second order neurons containing microfilaments and vesicular elements (open arrows) can be distinguished. Incurved arrow points to a lipid droplet. x 7,500

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these cells appeared heavily stained and they were mainly characterized by their ovoid or round profile and by a round nucleus with a large nucleolus (Fig. 11). Ultrastructural observations showed that cells forming clusters were in close apposition to each other (Figs. 12a,b). Their cytoplasm was electron-dense and contained well-developed Golgi apparatus, electron-dense mitochondria and numerous free ribosomes lying in the cytoplasm matrix between stacks of rough endoplasmic reticulum cisternae (Fig. 12a). The nucleus exhibited decondensed chromatin with little heterochromatin distributed in a thin border adjacent to the nuclear envelope. Centrioles were often seen in the most apical cells of the clusters, cells which also presented a supranuclear expansion of their cytoplasm (Fig. 12b, insert).

In parallel, the number of maturing receptor cells observed in the sensory epithelium of exposed fish increased with time exposure. Compared to maturing neurons described at 15 days, those seen at 30 and 60 days exhibited ultrastructural features characterizing the almost mature neurons with a dendrite close to, but not through the epithelial surface (Fig. 13a). Moreover, at 60 days, a few cells exhibited at the free surface a cytoplasm protusion of shaft-like shape containing centrioles and bundles of filaments and/or microtubules (Fig. 13b). However, in no case, cilia or microvilli could be detected. Unusual junctions of bead-like shape were also noted at the level of the constricted neck of these apical protrusions (Fig. 13b, insert).

A significant increase of goblet cells was noted with time exposure. As shown in Figure 11, goblet cells seemed to originate from cell clusters located in the lowest part of the sensory epithelium and no mitotic goblet cells were observed. An amazing feature of these cells was the accumulation in their apical pole of mucous droplets while cells had not yet begun or achieved their migration up to the epithelial surface.

In the olfactory nerve, the number of phagosomes in the ensheathing cells decreased after 30 days of exposure (Fig. 14) and at 60 days, they were replaced by large lucent vacuoles filled with a fibrillar network (Fig. 15). Concomitantly, the intact axons were more numerous after 30 and 60 days. In some cases, ensheathing cells presented an electron-dense cytoplasm showing a slightly irregular shape with a sheet-like extension that surrounded small bundles of olfactory axons or packed collagen fibres (Fig. 16).

In the olfactory bulb, the number of phagosomes in

glial cells though still large at 30 days decreased progressively, to disappear after 60 days. Dendritic growth cones filled larger areas of the glomerular neuropil. Some growth cones of olfactory axons became obvious after 60 days (Fig. 17a). The glomerular layer presented a more clear appearance mainly related to the presence of large translucent profiles. Electron-dense axon terminals establishing synaptic contacts were also observed at 60 days (Figs. 17b,c).

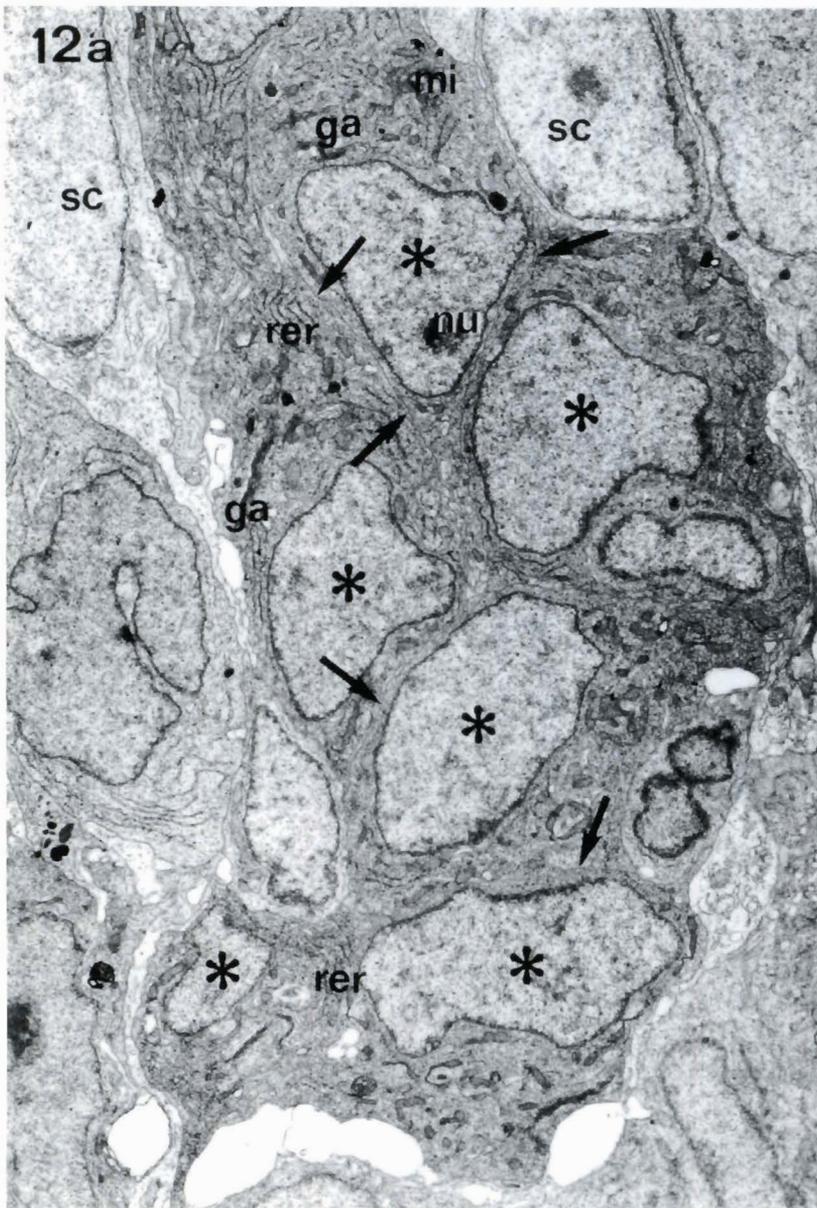
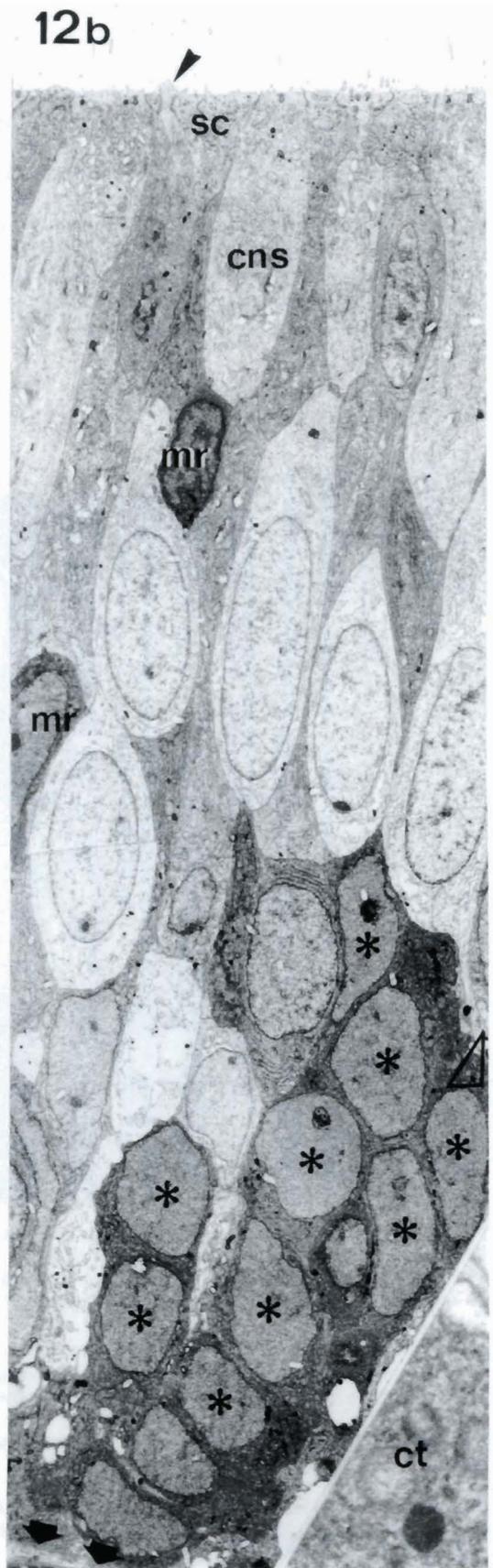
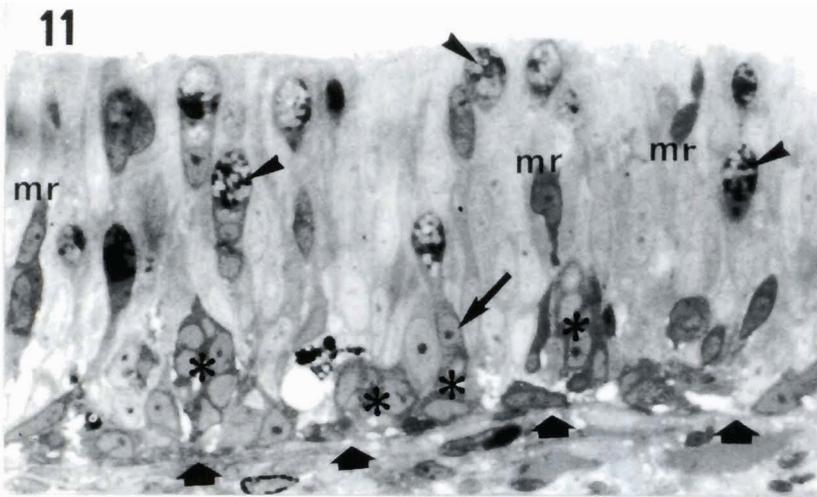
Discussion

Results of this study indicate that a 15-day exposure to copper, at a dose of 20 µg/l, caused specific damage to the olfactory receptor cells in adult rainbow trout. The heavy metal induced degeneration of all mature receptor cells as well as numerous immature neurons located in the lower part of the epithelium, whereas supporting and ciliated non-sensory cells remained almost intact. Such copper action upon the primary olfactory neurons has also been reported in trout exposed to moderate or high copper concentrations during short periods (Brown et al., 1982; Cancalon, 1982a; Hara et al., 1983; Klima and Applehans, 1990; Moran et al., 1992). As shown by scanning electron microscopy (Klima and Applehans, 1990), such an exposure condition may also affect the ciliated processes of non-sensory cells.

Contrary to what is reported in studies using high levels of copper, we have noted that the degenerating receptor cells observed at 15 days, did not exhibit signs of necrosis but rather typical features of a cell death by apoptosis. Degenerating receptor cells presented some nuclear condensation and segmentation, compaction of intact cytoplasmic organelles, loss of junctional complexes and cytoplasmic bleb formation pouring out of the epithelial surface, all of which represent typical morphological features of apoptosis described by Wyllie (1987). Even though such a pattern of apoptosis has never been described in the olfactory epithelium, it has been shown, however, in a micrograph of intact rat olfactory epithelium presented by Doucette et al. (1983a, Fig. 2) in which neuronal death by apoptosis could be found with neuron necrosis. In the sensory epithelium of control fish, no apoptosis could be seen, this probably being due to the fact that apoptosis is a scarce and transient process. In copper exposed fish, the presence of numerous apoptotic receptor cells strongly suggest that the metal at low concentration may act as a pathogenetic signal which initiates or enhances the onset of apoptosis

Fig. 11. Light microphotograph of a cross-sectioned lamella through the sensory epithelium of a fish exposed to copper for 60 days. Many clusters of cells (asterisks) are seen in the basal region of the epithelium. Arrowheads point to numerous goblet cells. Some of them seem to originate from cell clusters (arrow). mr = maturing receptor cell. x 1,100

Fig. 12. Electron micrographs of the sensory epithelium after 30 days (a) and 60 days (b) of copper exposure showing the ultrastructural features of the cell clusters. **a.** Cells forming the cluster (asterisks) are characterized by an electron-dense cytoplasm and are in close apposition to each other (arrows). x 5,200. **b.** Some cells of the cluster contain centrioles (ct: insert, x 22,500) in their supranuclear expansion. Arrowhead points to a cell exhibiting an unusual apical cytoplasmic protusion. cns = ciliated non-sensory cell, ga = Golgi apparatus, mi = mitochondria, mr = maturing receptor cell, nu = nucleolus, rer = rough endoplasmic reticulum, sc = supporting cell. x 2,700



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in these cells. This hypothesis does not seem unlikely since it is known that copper shows a high specific binding to DNA producing DNA strand breaks in several biological systems (Sagripanti et al., 1991). Such action of copper on DNA might be related to the peripheral condensation of nuclear chromatin described as one of the main features of apoptosis (Wyllie et al., 1980; Wyllie, 1987). Knowing that copper may also affect intracellular calcium homeostasis (Abramson et al., 1983; Kiss et al., 1991; Viarengo and Nicotera, 1991), one can suppose that this might play a role in apoptotic process since the rise of cytoplasmic Ca^{2+} with the

endonuclease activity has been reported to be an early signal for initiating apoptosis (Ojcius et al., 1991). On the other hand, no observation having been performed during the early stages of exposure, one cannot exclude the possibility that some receptor cell necroses might have occurred during this early period. Some receptor cell necrosis has been recently reported in brown trout after one day of exposure to low levels of copper (Moran et al., 1992). Experiments of short-term exposure to low copper concentration are in progress to verify if both patterns of cell death, apoptosis and necrosis, may be present in the olfactory epithelium in early stages of

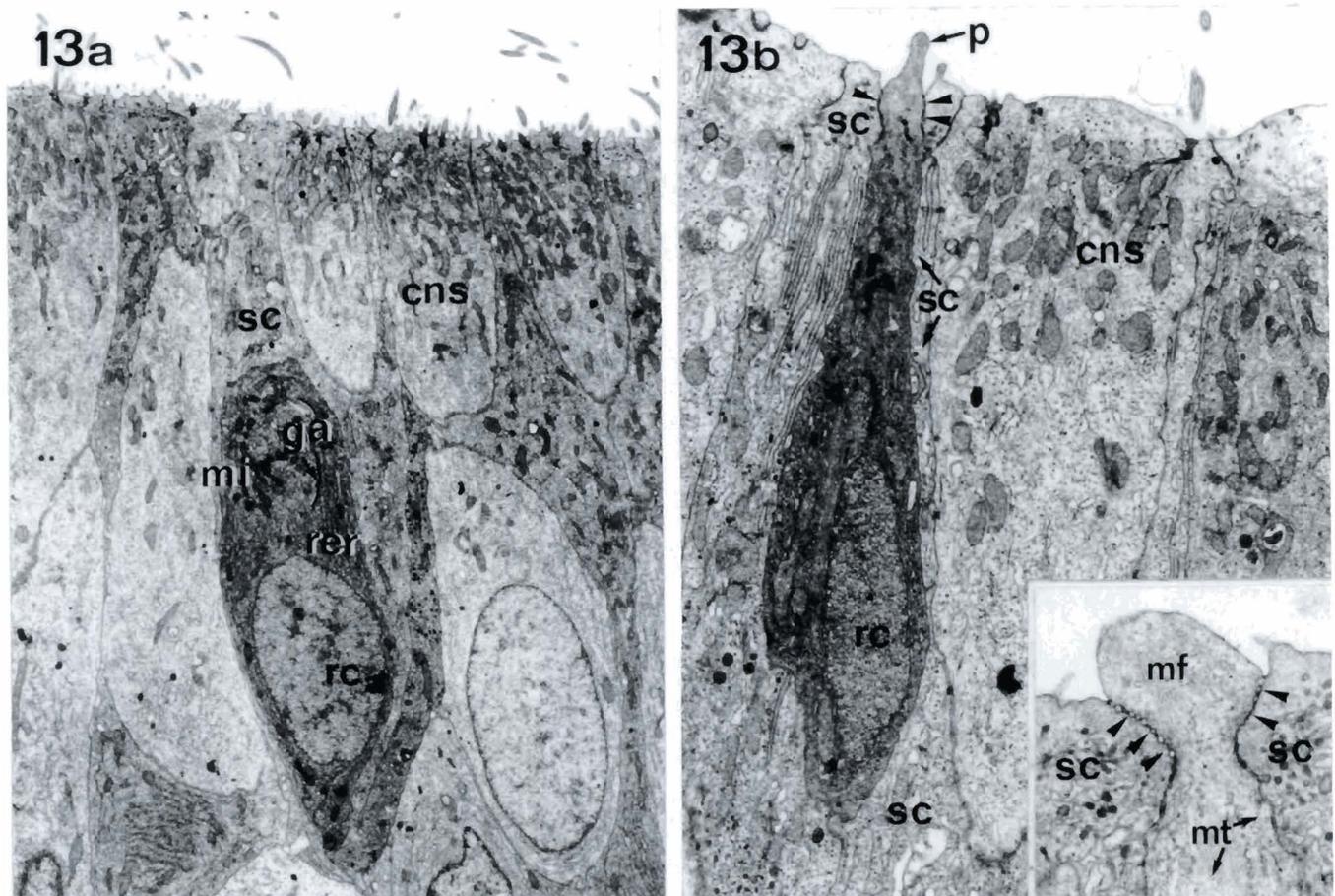
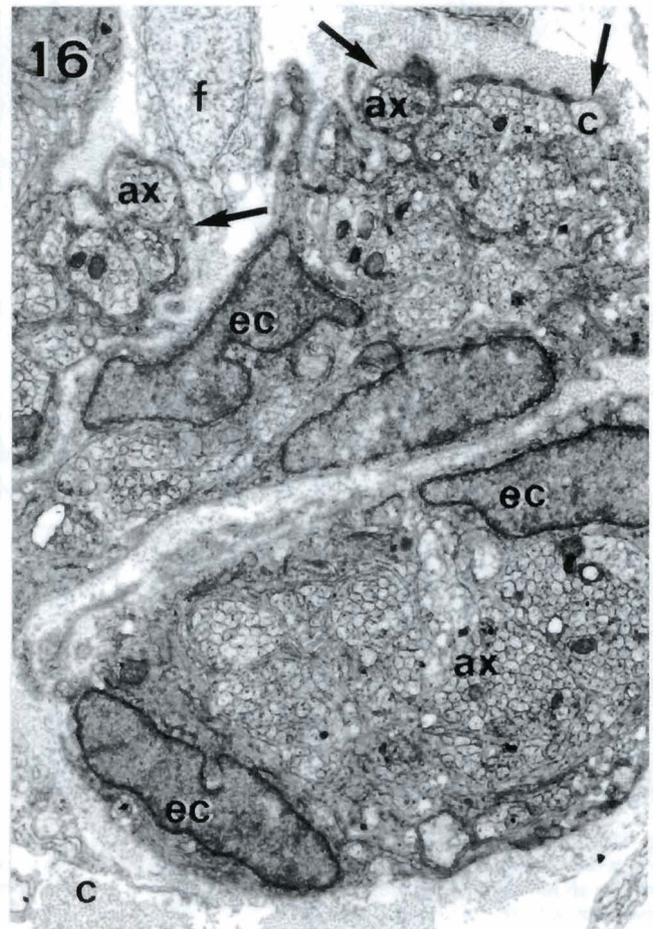
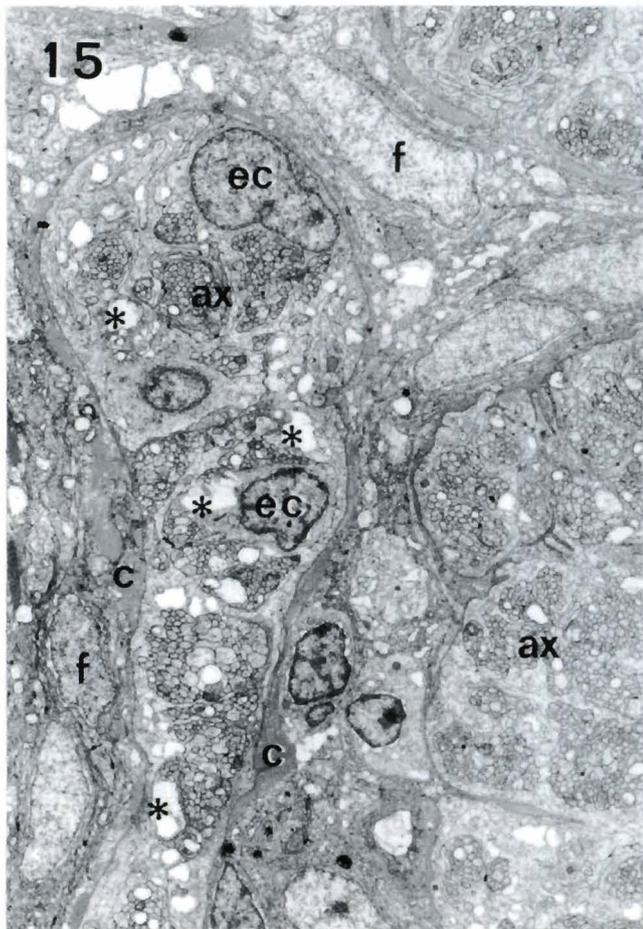
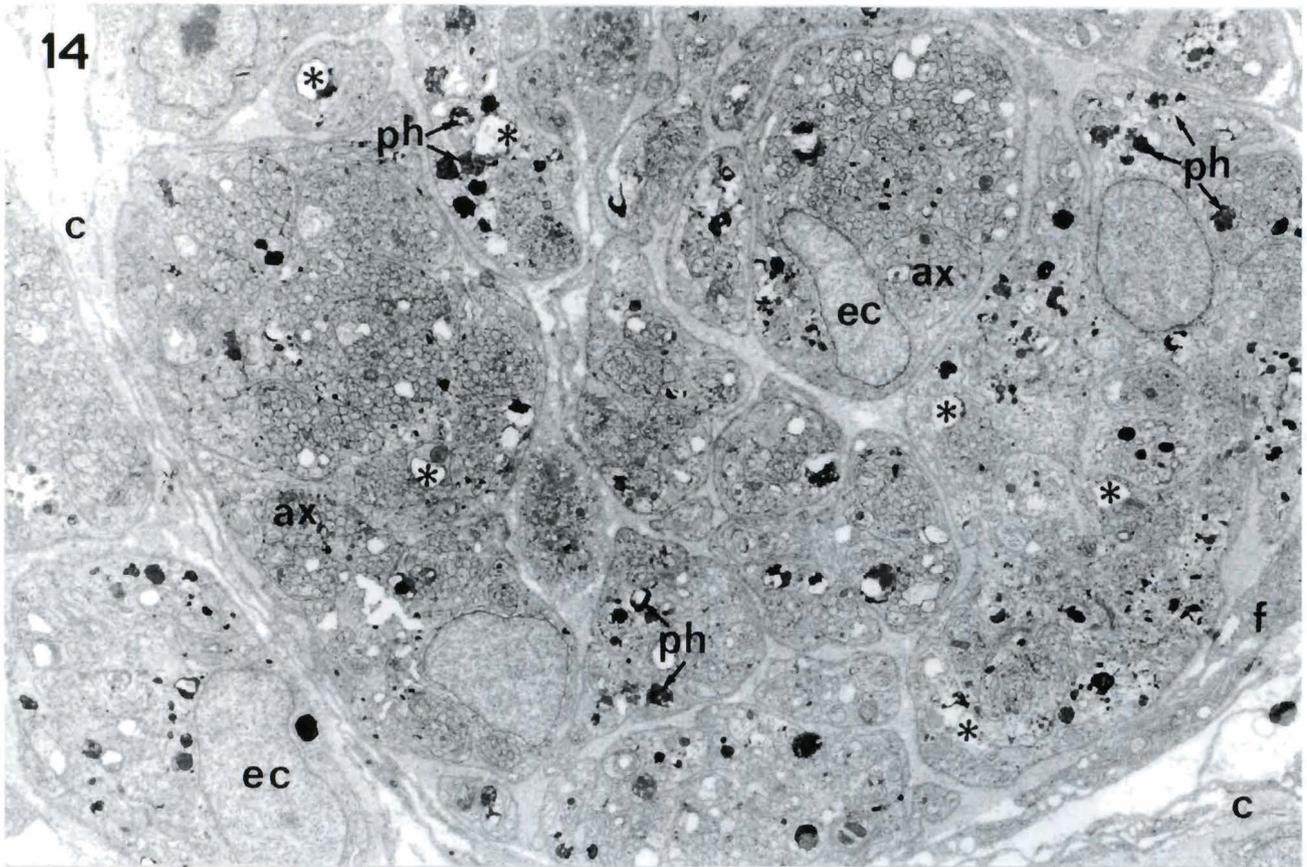


Fig. 13. Electron micrographs show the ultrastructural features of almost mature receptor cells (rc) found in the sensory epithelium of 30-day (a) and 60-day (b) exposed fish. In **b**, the receptor cell shown is characterized by a bare apical cytoplasm protrusion. See Fig. 12 for legends. a. x 3,100. b. 6,800. In the insert, unusual junctions (arrowheads) are observed at the level of the constricted neck of the apical protrusion. mf = microfilaments, mt = microtubules. x 13,500. cns = ciliated non-sensory cell, ga = Golgi apparatus, mi = mitochondria, rer = rough endoplasmic reticulum.

Fig. 14. In the olfactory nerve, the number of phagosomes (ph) appears reduced after 30 days of copper exposure. ax = axons, c = collagen fibres, ec = ensheathing cell, f = fibroblast, asterisks = clear vacuoles. x 5,000

Fig. 15. After 60 days of copper exposure, large clear vacuoles (asterisks) have replaced phagosomes in the olfactory nerve. x 3,600. See Fig. 14 for legends.

Fig. 16. The electron micrograph shows ensheathing cells (ec) in the olfactory nerve after 30 days of copper exposure. Some cells exhibit an electron-dense cytoplasm and a compact shape (lower part) while others expand slender sheet-like processes (arrows) around small bundles of axons (ax) or collagen fibres (c). x 7,200



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exposure and if so, to determine if they occur synchronously.

In spite of the degeneration of most receptor cells, the apparent structural integrity of the sensory epithelium noted after 15 days, appears mainly due to some hypertrophy of supporting cells. Vacant sites left by degenerated neurons were rapidly filled by hypertrophied processes of neighbouring supporting cells in such a way that the epithelial thickness remained relatively unchanged. No such hypertrophy of supporting cells was noted after olfactory nerve axotomy. This tissue reaction could be linked to our

experimental conditions showing the long lasting action of a low concentration of the heavy metal. In addition, supporting cells exhibited an increased number of ribosomes, rough endoplasmic reticulum and mitochondria, suggesting an increased metabolic activity which is, in normal conditions, already high in the olfactory epithelium (Cuschieri, 1974; Zielinski et al., 1988). This might be related to the fact that supporting cells seem involved in the phagocytosis of apoptotic receptor cells. As a consequence of copper exposure, at 15 days, one can also note in many supporting cells the presence of annulate lamellae which were not observed

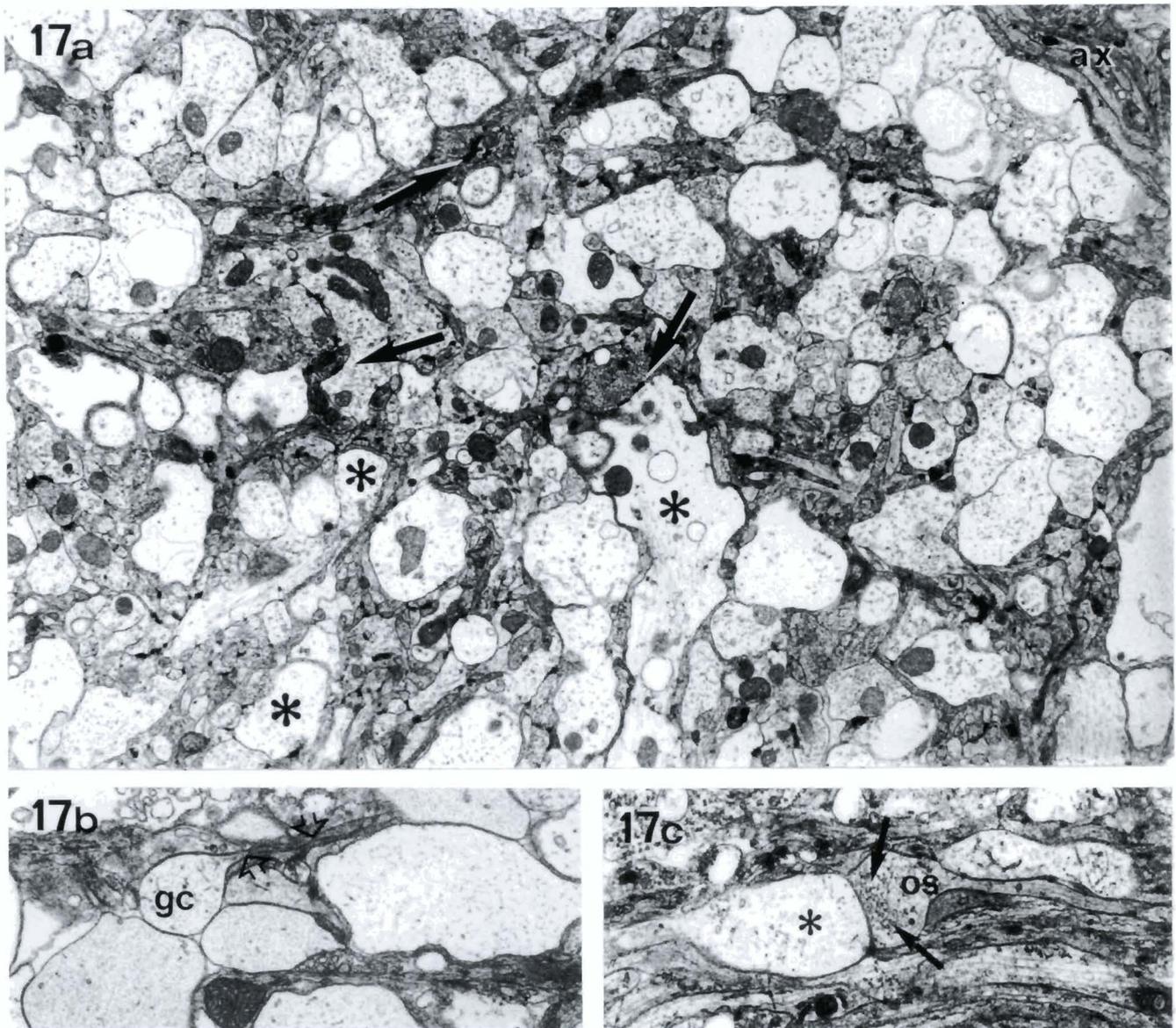


Fig. 17. The electron micrograph shows the bulb glomerular layer after 60 days of copper exposure. In **a**, asterisks indicate dendritic growth cones of second order neurons. Some electron-dense olfactory axons establish synaptic contacts (arrows). In **b**, a growth cone (gc) of an olfactory axon (open arrow) is shown. In **c**, the micrograph presents an olfactory synaptic ending (os) containing numerous synaptic vesicles (arrows) which contacts a dendritic growth cone (asterisk). a: x 7,500; b, c: x 11,400

after olfactory nerve axotomy. An enhanced development of annulate lamellae has been reported in response to heavy metal and toxic waste exposure in a variety of cell types (Kessel, 1992). These organelles being mainly seen at 15 days in the supporting cells, one can suppose that they could be somehow involved in the high metabolic activity of these cells during the time-course of receptor cell degeneration. This is in accord with the suggestion that annulate lamellae could play a role in synthesis of cellular components, such as mitochondria, tubulin and enzymes (Kessel, 1992).

The presence of numerous lipid droplets within supporting cells of exposed fish is probably also the reflection of the increased metabolic activity of these cells. We actually do not know if the presence of these inclusions is directly linked to the action of copper upon supporting cells or rather an indirect consequence of receptor cell death. A few studies (Enesco et al., 1989; Viarengo et al., 1990) have already demonstrated that heavy metals, including copper, may stimulate lipid peroxidation leading to formation of compounds accumulated in cells. One has to point out that the biggest lipid droplets were localized in the basal region of the olfactory epithelium, a region showing a high phagocytic activity. Moreover, when the phagocytosis activity is maximal at 15 days of exposure, lipid droplets were often seen associated with phagosomes in the ensheathing cells of the olfactory nerve as well as in astroglial cells of bulbar glomeruli. Similar results could be observed after olfactory nerve axotomy (see Cancalon, 1982b, Fig. 2; 1983, Fig. 7) or after SNC injury by irradiation (Maxwell and Kruger, 1965). Data suggest that the increase in lipid content might be regarded, at least in part, as a nonspecific response of glial cells to the injury of the olfactory system by copper. This assumption is reinforced by the fact that lipid droplets were no more present from the 30th day of exposure which corresponds to the period in which the process of receptor cell degeneration is completed.

Degeneration of receptor cells occurring during the early stage of exposure resulted in degeneration of numerous axons in the olfactory nerve and axon terminals in bulbar glomeruli. Ultrastructural features of degenerating axons resembled those described after nerve axotomy in mammals (Harding et al., 1977; Graziadei and Monti Graziadei, 1980; Doucette et al., 1983b). In parallel, one could observe a great phagocytic activity of both ensheathing cells in the lamina propria and astrocytes in glomeruli, thus allowing the removal of degenerated axons. These cells presented a reactive hypertrophy, but no obvious hyperplasia was noted. Such glial hypertrophy has also been shown after olfactory nerve section, but it was a transient event since the time-course of glial hypertrophy was closely related to that of receptor cell degeneration and regeneration (Anders and Johnson, 1990). Some glial hypertrophy was still reported at day 60, this probably being related to the fact that no complete morphological recovery of the sensory epithelium was observed in condition of

chronic exposure to the toxicant. We also have a few insights that new ensheathing cells were probably formed in the olfactory nerve during the time-course of exposure. At 30 and 60 days, some ensheathing cells exhibited an electron-dense cytoplasm, a morphological feature of maturing ensheathing cells described during mouse ontogenesis (Cuschieri and Bannister, 1975). This hypertrophy of glial cells in the lamina propria and the glomeruli as well as that of supporting cells in the sensory epithelium confirms that these cells are actively involved in the process of tissue scarring. Moreover, this reinforces the idea that supporting cells, contrary to ciliated non-sensory cells which remained unaffected, present some typical features of glial cells.

After 15 days of copper exposure, we still observed some intact axons in the olfactory nerve and axon terminals making synaptic contacts in the glomeruli. One can suppose that they belong to degenerating or newly dead receptor cells. This assumption does not appear unlikely since it is well known that axon degeneration following nerve crush or removal of mucosa does not occur simultaneously along the nerve, but rather progresses in a proximodistal direction (Cancalon and Elam, 1980; Cancalon, 1982b). In addition, as shown by Cancalon (1983), this axon degeneration seems to be temperature dependent, the velocity of olfactory axon degeneration in garfish (*Lepisosteus osseus*) being noticeably reduced at 10 °C compared to higher temperatures. Since fish were kept in low water temperature (4.6-5.8 °C) in the present experiment, this has probably slowed down the process of axon degeneration. On the other hand, one cannot exclude the possibility that some of these intact axons may belong to the population of differentiating receptor cells scattered in the sensory epithelium, a cell population corresponding to immature neurons which had remained unaffected by the toxicant. One can assume that these cells might be involved in a process of differentiation, including some axon growth which could be a precocious event since some basal cells were seen retrogradely labelled after an application of HRP to the cut of olfactory nerve in brown trout (Moran et al., 1992).

Despite the permanent exposure to copper, the olfactory epithelium presented some signs of neuronal regeneration during the time-course of the experiment. At days 30 and 60, one can note clusters of immature cells in the basal region of the sensory epithelium. These clusters resemble nest-like structures described in intact mouse olfactory epithelium (Graziadei and Monti Graziadei, 1979) or after axotomy (Monti Graziadei and Graziadei, 1979). As in the mouse, most of the elements forming the clusters in exposed fish presented the morphological features of immature neurons. The more apical neurons showed a dendrite-like stump at their distal pole and sometimes contained centrioles. The presence of such cell clusters is certainly the reflection of an increased mitotic activity in the basal region of the epithelium similar to that reported after the complete

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disappearance of mature receptor cells following axotomy or bulbectomy (Graziadei, 1973; Monti Graziadei and Graziadei, 1979; Simmons et al., 1981; Evans et al., 1982; Schwartz Levey et al., 1991; Carr and Farbman, 1992). In mammals, two subpopulations of basal cells, dark and light or globose cells have been described (Graziadei and Monti Graziadei, 1978, 1979), and recent data suggest that only the globose basal cells might proliferate following bulbectomy or axotomy (Schwartz Levey et al., 1991; Suzuki and Takeda, 1991). The two subpopulations of basal cells described in mammals could not be distinguished either in controls or in exposed fish.

It seems likely that the majority of the almost mature neurons seen by day 30 with a dendrite close to, but not through the epithelial surface, originated from the cell clusters. Even though their number increased with time exposure, one has to note that no fully mature neurons, characterized by an olfactory knob provided with cilia or microvilli, could be found even at 60 days. The time-course of receptor cell regeneration is rather comparable to that reported by Zielinski and Hara (1992) following olfactory nerve section in rainbow trout. They have shown that the first dendrites reached the apical surface between the 42th and the 56th day post-axotomy and that fully mature receptor cells were detected by day 76. It could be noted that faster time-courses of primary neuron regeneration could be observed with higher water temperatures (Moran et al., 1992). On the other hand, if we had extended the copper exposure for a while, it could be asked whether fully mature receptor cells might have at a certain time emerged at the epithelial surface. It is also possible that due to the toxicant action, cells would have remained at the maturing stage described above until the copper exposure ends. Another possibility would be that cilia and microvilli of the dendritic knob might be eliminated as fast as they appear.

Also reported at 30 and 60 days of exposure, was the proliferation of goblet cells in the sensory epithelium. Comparable goblet cell hyperplasia has been shown in the olfactory organs of trout alevins in response to long lasting exposure to copper (Saucier et al., 1991). There is agreement that the increased production of mucus resulting from a goblet cell hyperplasia, as shown here or from cell hypertrophy (Baker, 1969; Eisler and Gardner, 1973; Gardner, 1975; Lock and van Overbeeke, 1981), is a classical response to the action of toxicants. According to Bertmar (1972), goblet cells seem to originate from stem cells located in the basal region of both sensory and indifferent epithelia. It has also been reported that some mitosis might still occur in the midway of the epithelium. No such goblet cell mitosis was seen in the present study. Our data rather indicate that goblet cells originated from the clusters of cells which apparently appear synchronously with the goblet cell proliferation. From this, one can suggest that the basal cells might be pluripotent cells giving rise to at least two progenitor populations, one differentiating into

receptor cells and the other into goblet cells.

At the bulbar level, some synaptic contacts between axon terminals and second order neurons could be observed all along the exposure period. Axodendritic synapses seen at 15 days could be related to axon terminals belonging to unaffected, degenerating or newly dead receptor cells, whereas the presence of synapses at 30 and 60 days of exposure is more puzzling. It could be asked if they were made of either not yet degenerated or newly formed olfactory axons. The first possibility seems unlikely since at 30 days and even before, no more degenerating receptor cells were seen in the sensory epithelium. At day 60, the presence of growth cones on olfactory axons ending in glomeruli might indicate that some process of glomerular reinnervation by growing axons of newly formed receptor cells might occur. In addition, as for bulbar reinnervation noted after axotomy (Graziadei and Monti Graziadei, 1980), glomerular neuropiles exhibited some typical features of embryonic material described by Hinds and Hinds (1972). This assumption is not reinforced, however, by data in mammals showing that during embryogenesis, the first axodendritic synapses begin to develop after the final stage of dendritic terminal maturation of and ciliogenesis in receptor cells (Farbman, 1991). Experiments of retrograde labelling using a tracer like HRP should be useful to clarify this point. Thus, after an injection of HRP into the bulbar glomeruli of 60-day exposed fish, it should be possible to verify if some receptor cell populations are connected to the olfactory bulb and if so, to determine which of the cell clusters and/or the almost mature cells, are involved.

The results well demonstrate how the olfactory system manages to adapt to the environmental stress of a long lasting exposure to low levels of copper. After the death of the vulnerable olfactory receptor cells and their removal by neighbouring cells, one could observe an increased mitotic activity in the basal region of the epithelium as well as some process of primary neuron differentiation in the upper part of the epithelium. Thus, even though the exposure to the toxicant went on, some homeostasia seemed to be maintained in the olfactory system which also indicates that a complete morphological recovery should occur rapidly if fish were returned to well water. Nevertheless, even though fish seem to acclimate, one has to be worried about the olfactory function and the olfaction-mediated behaviours during the time-course of exposure. This all the more that no fully mature receptor cells were seen emerging at the epithelial surface during the exposure period. We have a few indications however, that the olfactory function though operational is greatly reduced, since adult trout maintained in the same experimental conditions for several months, stayed hyposmic as long as they were exposed to the heavy metal (Saucier et al., in preparation). As far as this reduction of sense of smell is a consequence of a chronic exposure to a copper level just above the standard nontoxic value, one may easily extrapolate what could be the biological impact of

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exposures to higher toxicant levels as often reported in studies of environmental toxicology.

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