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Localised degeneration occurs in aged mouse olfactory epithelium

Olfactory neuroepithelium of adult vertebrates retains a population of basal stem cells capable, throughout life, of dividing and differentiating into mature olfactory receptor neurons (see Farbman, 1994). The rate of turnover of olfactory receptor neurons is influenced, in part, by environmental conditions (Hinds et al. 1984). The olfactory system is known, however, to undergo a general decline in function with age. Age-related changes have been described in the rat olfactory bulb, with a decline in size in later life (Hinds & McNelly, 1981). However, this was thought to be secondary to changes within the olfactory epithelium as changes in the number of olfactory receptor neurons directly influence the size of the olfactory bulb. Thus the primary deficit in declining olfactory function may reside in the olfactory epithelium. Studies in humans demonstrated that olfactory function diminishes with increasing age (Ship et al. 1996), suggesting a possible decrease in the number of olfactory sensory neurons. Although the structure of both developing and mature olfactory epithelium (Farbman, 1994) have been well characterised, less information exists on changes relating specifically to ageing. A reduction in thickness of the olfactory epithelium was evident in ageing humans, with a concomitant loss of the normal zonal distribution of supporting and sensory cell nuclei, a less well defined boundary between respiratory and sensory epithelia, and an increase in pigment granules within the supporting cells (Naessen, 1971). Similar lysosome-like inclusion bodies were also noted in olfactory epithelium of adult rabbits (Mulvaney, 1971) and dogs over the age of 17 y (Hirai et al. 1996). Decreases both in the total area of olfactory epithelium and in dendritic knob density have been described in rats aged 29 mo and above (Hinds & McNelly, 1981). This is unlikely to be due to a loss of regenerative capacity since, in hamsters, regeneration of olfactory receptor neurons is observed in aged animals (Morrison & Costanzo, 1995). A scanning electron microscopic comparison of young and old rat olfactory epithelium revealed changes in membrane-limited dense bodies in olfactory receptor neuronal cell bodies (Naguro & Iwashita, 1992). In old rats (21 and 36 mo), large irregular bodies were found in the supranuclear area of olfactory receptor neurons and were also abundant throughout the sustentacular cells, resulting in cell enlargement and the loss of about 2 layers of olfactory receptor neuron perikarya. They contained lipid droplets and numerous irregular osmiophilic lamellae resembling lipofuscin granules (Naguro & Iwashita, 1992). No attempt was made to quantify these age related changes. The present study aimed to detail fully the morphological changes in the aged mouse olfactory epithelium and is a continuation of a previous pilot study (Appleton et al. 1996).

Male CBA mice were maintained under normal animal house conditions. Animals aged 6–10 mo, 26 mo, and 30–34 mo (4–6 per age group) were deeply anaesthetised with sodium pentobarbitone (Sagatal) and perfused through the left ventricle initially with mammalian Ringer solution followed by 3% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer (PB). The heads were cut in a parasagittal plane, and the septum and nasal conchae carefully dissected. For light and electron microscopy, the conchae and septum were decalcified in 10% EDTA in 0.1 M PB for 2–3 h, followed by 3 15 min buffer rinses and 1 overnight rinse. The tissue was then postfixed in 1% osmium tetroxide, dehydrated in a graded ethanol series to absolute ethanol, and transferred to propylene oxide prior to infiltration with Araldite for polymerisation at 60 °C. Semithin 1 μm sections were routinely stained in 1% toluidine blue for light microscopy, whilst 60–80 nm sections of selected areas were stained with uranyl acetate and lead citrate for transmission electron microscopy. Various histochemical stains were also employed to investigate the observed inclusions further. (1) Sections were immersed for 20 min in 0.05% Nile Blue A in 1% sulphuric acid, and then either briefly rinsed with 1% sulphuric acid and dehydrated and mounted in Histomount, or washed in running water for 10–20 min and mounted in glycerine jelly (with the former modification, lipofuscin should appear brown and with the second method, dark blue or green). (2) For the Schmorl method sections were immersed for 5 min in ferricyanide solution (1% ferric chloride and 1% freshly prepared potassium ferricyanide), washed in tap water, dehydrated and mounted in Histomount. Substances which reduce ferricyanide (e.g. melanin and lipofuscin) should appear dark blue as well as tissue components containing active sulphydryl groups.

Comparison of toluidine blue stained olfactory epithelium from adult and aged animals revealed striking differences in morphology. The olfactory epithelium of 6 mo mice displayed characteristic features (Fig. 1*a*). Many layers of cells were present, with the most superficial layer containing typically pale oval sustentacular cell nuclei surrounded by cytoplasm. The olfactory dendritic knobs were visible lying on the surface. In 3 of the 30–34 mo animals (n = 4), regions of the olfactory epithelium, of both the nasal septum and conchae, were found to contain striking large intracellular accumulations of uniformly stained, amorphous material. Indeed transverse sections from this age group clearly demonstrated 2 types of olfactory epithelial morphology, frequently observed on opposite sides of the septal cartilage or conchae, with one surface covered by an epithelium comparable to that of 6 mo animals, whilst on the other surface it was filled with large, darkly stained amorphous inclusions (Fig. 1*b*). Inclusions were irregular in size and shape but formed discrete units, suggesting that they were membrane limited. A substantial reduction in the number of nuclei was noted in affected areas, at all levels within the epithelium (i.e. there appeared to be a loss of sustentacular cell nuclei as well as olfactory receptor neuron nuclei) and in olfactory knobs, compared with the unaffected area of olfactory epithelium on the opposite side of the septum. In

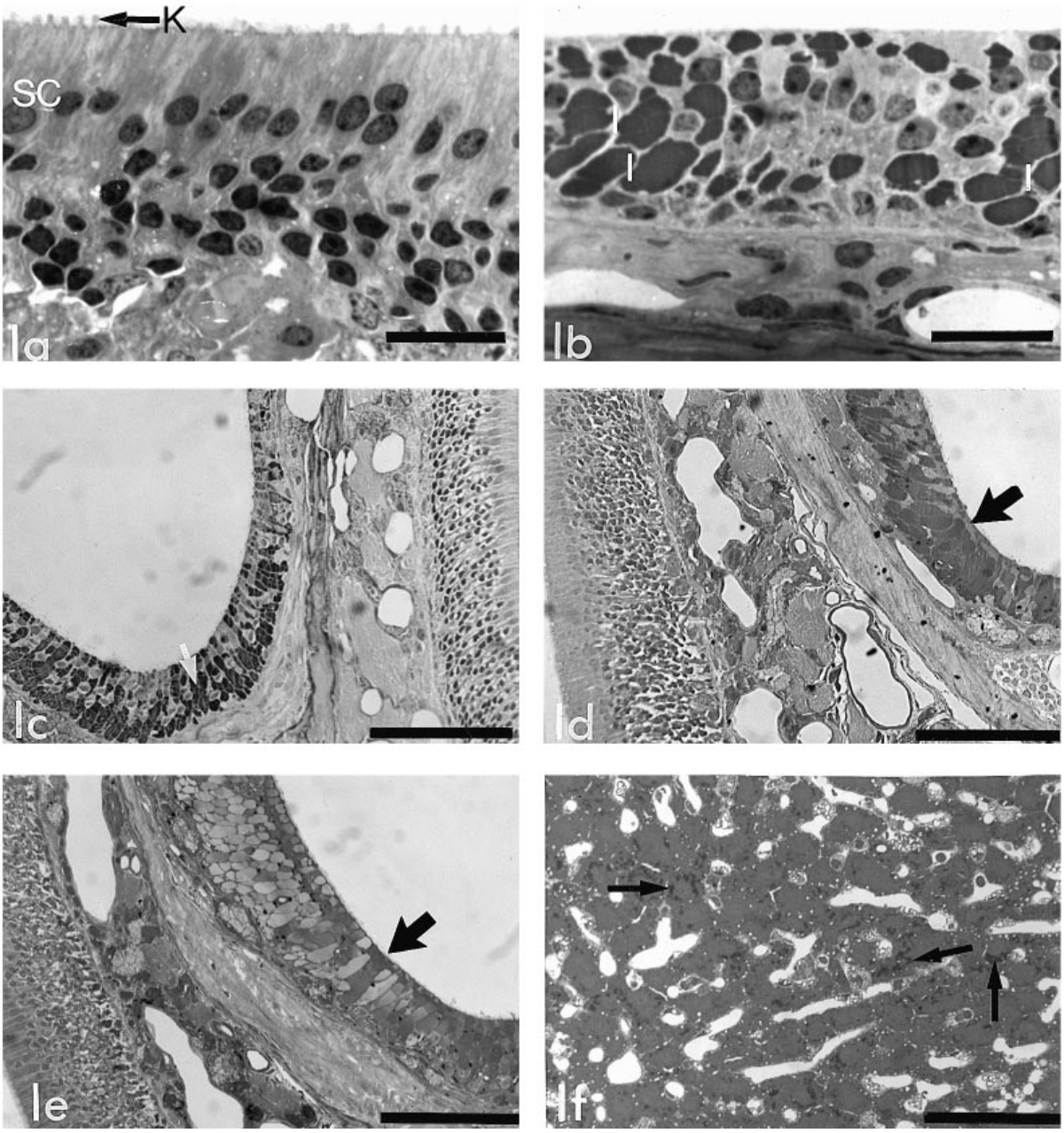

Fig. 1. (*a*) and (*b*) are 1 μm resin sections stained with toluidine blue of olfactory epithelium (OE) from (*a*) 6 mo conchae showing the superficial layer of sustentacular cell nuclei (SC) and an underlying 3–4 layers of nuclei of receptor neurons at different developmental stages. Dendritic knobs (K) are readily visible, and (*b*) 34 mo septum lined with affected OE with large inclusions (I) and few nuclei; (*c*), (*d*) and (*e*) are 1 μm resin sections of 30 mo conchae stained with Nile blue (*c*), dehydrated in acetone, (*d*) Nile Blue, nondehydrated and (*e*) the Schmorl method. Both affected (arrows) and unaffected areas of OE can be seen. 34 mo mouse liver was stained by the Schmorl method at the same time (*f*). Note the very dark staining in the hepatocytes (arrows) indicating the presence of lipofuscin. Bars: *a*, *b*, 20 μm; *c–f*, 100 μm.

many regions the affected olfactory epithelium appeared qualitatively thinner than the unaffected olfactory epithelium. The proportion of olfactory epithelium affected by the inclusions varied greatly, in 2 cases being a single patch of between 300 and 700 μm in length whilst in 1 instance, 3 separate affected areas were evident per section. Between 4 and 37% of the epithelial surface area in the tissue sections was estimated to be affected. There was no evidence of such accumulations of inclusions in olfactory epithelia from either the 6–10 mo (n = 6) or from the 26 mo (n = 5) animals. It is possible, however, that the inclusions were present in the 26 mo group but in smaller undetectable form. Although 26 mo is considered aged for mice, the maximum lifespan of a laboratory mouse fed ad libitum is around 36 mo (Moore et al. 1995), and the 30–34 mo age group are therefore considered to be of extreme age.

The granules reported to accumulate in ageing human olfactory epithelium were relatively small (< 1 μm), osmophilic and termed pigment granules (Naessen, 1971). Large, irregularly shaped granules described by Naguro & Iwashita

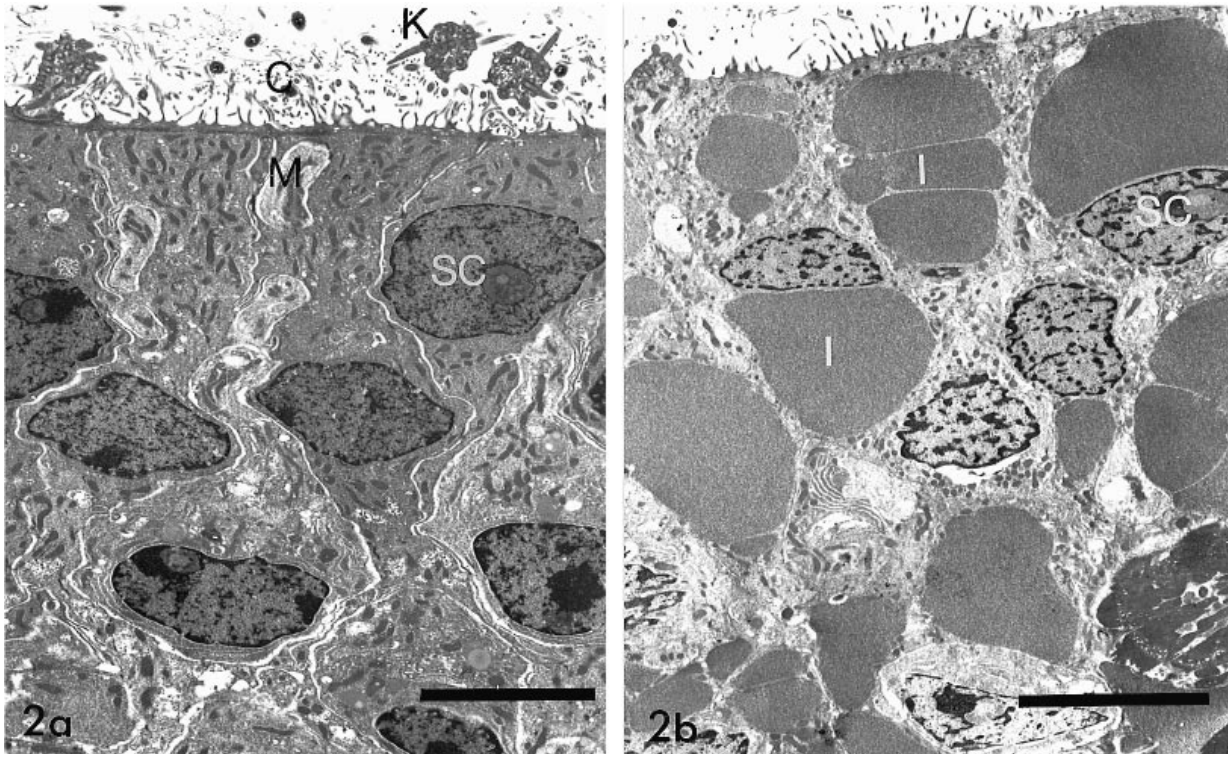

Fig. 2. (*a*) 6 mo olfactory epithelium. Note the olfactory dendritic knobs (K) and the layer of cilia (C) and the elongated mitochondria within the dendrites (M); (*b*) 34 mo affected olfactory epithelium. Note the abundant granular inclusions (I), the crystalloid structure (lower right) and the apparent coalescence of inclusions within sustentacular cells (SC). Bars, 5 μm.

(1992) were unique to aged rat olfactory epithelium; they were thought to be composed of ageing pigment lipofuscin. Although in general much smaller than those we have identified (< 2 μm) it was possible that the large inclusions in the present study were composed of a form of lipofuscin which accumulates in ageing cells, and in particular those which have a low turnover rate such as the sustentacular cells. An attempt was therefore made to identify lipofuscin within the olfactory epithelium of aged animals. Lipofuscin is a natural brownish pigment which exhibits autofluorescence. However, observation of unstained 1 μm sections of 34 mo olfactory epithelium, containing large quantities of inclusions, had no natural pigmentation and did not exhibit autofluorescence. Nile Blue and ferricyanide (Schmorl method) are both routinely employed to stain lipofuscin and other natural pigments such as melanins. With Nile Blue 2 modifications of the staining method were used. In the first, sections were dehydrated in acetone, which resulted in a pale cytoplasm with darker nuclei and, intensely stained inclusions (Fig. 1*c*), and although initial toluidine blue staining suggested the inclusions to be homogenous in nature, heterogeneous staining characteristics were revealed with some much darker than others, and with some regions of individual inclusions darker than other neighbouring areas (arrow in Fig. 1*c*). In the second method the dehydration step was omitted with sections mounted directly in glycerine jelly to give less nuclear staining and green stained inclusions (arrow in Fig. 1*d*). Thus although the inclusions stained with Nile Blue it was not with the characteristics of lipofuscin. The heterogeneity was more apparent using the Schmorl method, indicative of variation in the composition of individual inclusions (Fig. 1*e*). The possibility of small deposits in 26 mo olfactory epithelium was investigated, but inclusion material remained undetectable using this technique. As a staining control, 34 mo mouse liver was included which in this case resulted in intensely dark stained granular material, typical of lipofuscin, evident within the hepatocytes (Fig. 1*f*). Thus, despite the limitations of using resin sections, the results suggest that the large inclusions in aged olfactory epithelium, were not typical lipofuscin, a finding which is consistent with those reported by Mulvaney (1971) for inclusions in adult rabbit olfactory epithelium. The precise chemical nature of the large inclusions remains to be fully elucidated.

TEM studies enabled a more detailed examination. Figure 2*a* illustrates the normal features of adult (6 mo) olfactory epithelium. Olfactory dendritic knobs with profuse cilia were abundant on the surface, whilst the apical cytoplasm of the sustentacular cells contained numerous mitochondria, and there were many microvilli. Elongated mitochondria of the receptor neuron dendrites (Naguro & Iwashita, 1992) were obvious. Sections of the extreme aged (34 mo) material (Fig. 2*b*) revealed the inclusion material to be homogenous, relatively nonosmiophilic and predominantly granular. At the ultrastructural level the material was more characteristically proteinaceous rather than lipidic. It is possible that this is a form of phagocytic debris, with the occasional crystalloid inclusion within the olfactory epithelium suggestive of macrophage activity (Marshall et al. 1988). Phagocytic cells have indeed been described in the rat olfactory epithelium and after experimental manipulation an initial small inflammatory response with an influx of macrophages has been reported (Suzuki et al. 1995). Another possibility is

that the inclusions result from accumulation of material synthesised by the sustentacular cells which has failed to be released. In this case, however, it would perhaps be expected to be present in abundant vesicles rather than the large inclusions. The inclusions clearly ranged in size and shape and appeared to be coalescing, a feature not characteristic of lipofuscin. In badly affected areas inclusions were found throughout the thickness of the epithelium with large accumulations at the level of the basal cells. Although the sustentacular cell nuclei were still present in the superficial nuclear layer, the number of receptor neuron nuclei was greatly reduced in affected areas, and indeed the basal cell layer was often disrupted suggesting a consequent reduction in stem cell numbers. The number of olfactory knobs observed on the epithelial surface also diminished. Occasional patches of respiratory epithelium were affected; with cells containing amorphous material similar to that found in the olfactory epithelium.

An obvious qualitative reduction in the number of olfactory knobs as well as the number of nuclei, was evident in the morphological observations, indicating a reduction in the number of both mature and immature olfactory receptor neurons in aged animals in areas affected by the inclusion material. In an effort to confirm this, both the number of olfactory knobs (to ascertain loss of mature receptor neurons) and the thickness of the olfactory epithelium (indicating loss of total cell numbers) were scored, at the LM level, in affected areas from 30–34 mo animals and compared with values from the adult (6–10 mo), 26 mo animals and from unaffected areas in the 30–34 mo olfactory epithelium. Olfactory knob numbers per 100 μm in unaffected areas, i.e. lacking inclusions, of 30–34 mo olfactory epithelium did not differ significantly from that of 6 mo or 26 mo animals, with counts of: 22.3 ± 2.2, 22.7 ± 1.3 and 22.8 ± 1.2, respectively. However, in affected areas containing inclusions from 30–34 mo animals, the number of olfactory knobs was significantly reduced to 6.1 ± 2.2 ($P < 0.001$, ANOVA). Measurements of the thickness of the olfactory epithelium gave a similar finding: the mean thickness of olfactory epithelium in unaffected areas from 30–34 mo, adult 6 mo, and 26 mo animals was 50.4 ± 2.3 μm, 56.8 ± 1.6 μm and 54.5 ± 2.3 μm respectively. Again, none of these values were significantly different. However, the affected areas of olfactory epithelium in 30–34 mo animals were significantly thinner at 32 ± 3.7 μm ($P < 0.001$). Reports exist in humans of a reduced thickness of the aged olfactory epithelium due to receptor neuron loss (Naessen, 1971). Hinds & McNelly (1981) have reported quantitative evidence for receptor neuron loss in the aged rat olfactory epithelium, occurring only relatively late in life: consistent the quantitative findings described here. The surrounding olfactory epithelium in 30–34 mo samples was not significantly different from either the 26 mo age group or the 6–10 mo animals. Thus, even in extreme aged animals the majority of the olfactory epithelium was apparently intact indicating that olfactory deficit associated with ageing is in part due to localised severe tissue degeneration rather than a general decline in receptor neurons. This is consistent with the findings of Morrison & Costanzo (1995) who demonstrated that the capacity for receptor neuron recovery and reconnection persists in the hamster well into old age.

In conclusion this report highlights novel quantitative structural changes in aged mouse olfactory epithelium. Future studies should shed light not only on the significance of the inclusions and on factors controlling receptor neuron turnover, but may also provide an insight into the ageing process.

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