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Metallothioneins 1 and 2 Are Expressed in the Olfactory Mucosa of Mice in Untreated Animals and during the Regeneration of the Epithelial Layer

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We have examined the expression of the MT1 and MT2 isoforms of metallothionein in the mouse olfactory mucosa. In untreated mice, metallothionein was strongly expressed in supporting cells, acinar cells of the Bowman's glands, and olfactory neurons. Expression was however restricted to a subset of cells within each type, and to zones within the olfactory system. Irrigation with $ZnSO_4$ solution caused exfoliation of the olfactory epithelium and during the resultant regeneration, metallothionein immunoreactivity was associated with the proliferating basal cells. The ability to express MTs 1 and 2 did not appear to be obligatory for the early stages of regeneration since mice which do not express these isoforms responded similarly to wild type mice. Strong nuclear expression of metallothionein was noted in the untreated olfactory chamber following unilateral irrigation.

Metallothioneins (MTs) are small cysteine-rich proteins which are found in all animals [11]. There are four isoforms of MT in mice, MT1 and 2 which are expressed in most tissues, whilst MT3 [12] and MT4 [17] are restricted to neural tissue and squamous epithelium respectively. MTs bind heavy metals like zinc, copper and cadmium, suggesting that they might be involved in the metabolism or detoxification of these metals, or alternatively, recent evidence suggests that MTs may form part of the antioxidant defences of the cell [4]. The neural expression of MT is topical: MT1 and 2 isoforms are predominantly expressed in astrocytes, but MT3 protein and mRNA is reported to be found both in astrocytes [23], and neurons [12] respectively. MT3 is a neuronal growth inhibitory factor which is thought to be down regulated in humans during Alzheimer's disease [23].

The expression of MT during the regeneration or hypertrophy of tissues is elevated, for example following partial hepatectomy [21, 22] and in the remaining kidney following unilateral nephrectomy [24]. In the former case, MT immunoreactivity is localised into the nucleus of hepatocytes, suggesting that it might play a functional role at this location. To extend these studies to another type of tissue capable of undergoing functional regeneration, we have examined the expression of MT in the mouse olfactory mucosa. The olfactory epithelium contains a number of well defined cell types of different functional properties e.g. supporting cells, sensory olfactory neurons and the continuously dividing basal cells which form the precursor cells of olfactory neurons [5]. The lamina propria is occupied by Bowman's glands and these contribute to the mucus layer of the olfactory epithelium This continually regenerating tissue is exposed to environmental insults and is likely to have developed appropriate protective mechanisms. This report examines the expression of MT both in the untreated mouse olfactory mucosa, and following zinc sulphate irrigation, which leads to exfoliation and then, regeneration, of the olfactory epithelium.

MATERIALS AND METHODS

Mice used were six week old males housed under standard conditions. All procedures were approved by the University of Tasmania Animal Ethics Committee. Strains used were C57B1/6J or a transgenic line which lacks functional MT1 or MT2 genes [13]. Zinc sulphate irrigation of mice was performed as described previously [6]. RNA was isolated from appropriate tissues and subjected to RT-PCR using standard techniques [19] with primers designed to bind to the 5′ and 3′ non-coding regions of MT2, MT3 and MT4 respectively.

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TABLE 1

Colocalisation of MT Subform mRNAs and Immunoreactivity in Wildytpe (C57) and MT-Deficient Mouse Tissues ($MT^{-/-}$)

		Liver		Brain		Olfactory epithelium		Olfactory bulb		Tongue epithelium	
Strain/gene		mRNA	Immuno	mRNA	Immuno	mRNA	Immuno	mRNA	Immuno	mRNA	Immuno
C57	MT2	Yes		Yes		Yes		Yes		Yes	
	MT3	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	MT4	No		No		No		No		Yes	
$MT^{-/-}$	MT2	No		No		No		No		No	
	MT3	No	No	Yes	No	Yes	No	Yes	No	Yes	No
	MT4	No		No		No		No		Yes	

Note. Gene specific mRNAs were detected in each tissue using RT-PCR, whilst immunostaining was performed with a monoclonal anti-MT antibody.

Zinc sulphate irrigation was performed according to the method described previously by Chuah et al., 1995 [6]. Prior to treatment, mice were anesthetised with 2.4% Nembutal (30 mg/kg). Experimental mice were administered 100 μl of 175 mM $ZnSO_4$, while the control mice received 100 μl of physiological saline (0.9%). A 1 ml teberculin syringe, fitted with a number 27 needle that had been filed smooth was used to introduce the solutions to the nasal cavity. After the appropriate treatment times, tissues were removed and fixed in Bouin's fixative, dehydrated in alcohols, cleared in chloroform and embedded in paraffin wax. Immunohistochemistry was performed using the enzyme-labelled streptavidin technique [8], as described previously [9]. Antibodies and dilutions used were as follows: monoclonal anti-MT antibody obtained from Dako (1:50); anti-olfactory marker protein (OMP) antiserum obtained from Dr Frank Margolis (1:500); and anti-proliferating cell nuclear antigen (PCNA) antibody obtained from Zymed Laboratories Inc (1:100). Secondary antibodies, and horseradish peroxidase conjugated streptavidin were from Biogenex. Peroxidase activity was visualised with 3,3′ diaminobenzidine (0.4%) and hydrogen peroxide (0.015%). Stained sections were mounted in D.P.X before photography.

RESULTS AND DISCUSSION

The expression of metallothionein was observed using a commercially available antibody (E9, Dako). To determine its specificity for each MT isoform, the distribution of anti-MT immunostaining in a panel of tissues was correlated with the presence of isoform specific mRNAs, as determined by RT-PCR. Furthermore, both wildtype (C57Bl/6J) mice, and transgenic mice which do not express MT1 or MT2 ($MT^{-/-}$) [13] were used (Table 1). For example, brains from wildtype mice expressed mRNA from MT2 and MT3. MT1 mRNA was not examined because it has been shown previously that MT1 and MT2 genes are coordinately expressed [20]. As expected, MT-deficient-mice ($MT^{-/-}$) expressed only MT3 mRNA in the brain. Similar experiments were conducted for liver, olfactory bulb, tongue and olfactory epithelium and confirmed earlier reports, namely that MT2 mRNA was expressed in all tissues of wildtype mice, MT3 mRNA was found in the brain and olfactory bulb of both strains, whilst mRNA for MT4 was restricted to tongue epithelial tissue. MT3 mRNA was unexpectedly found in the tongue and olfactory epithelia.

Secondly, histological sections from these tissues were immunostained for MT using the monoclonal antibody. Table 1 shows that immunoreactivity was always associated with MT2 mRNA in the wildtype mice, but that no immunoreactivity was observed in the brains or tongue epithelium of MT-deficient mice (primary data not shown). It can therefore be deduced that in the mouse, the antibody cross-reacts with MT2 and/or MT1 protein, but it either does not cross-react, or cross-reacts below the limit of detection, with MT 3 and 4 protein.

MT immunoreactivity was examined in coronal sections of the olfactory system of untreated, six week old wildtype mice. No immunoreactivity was observed when the primary antibody was omitted (Fig 1A), or when the antibody was preadsorbed with a mixture of MT1 and MT2 protein before use (not shown). Fig 1B shows extensive immunostaining in the olfactory epithelia and the lamina propria. Supporting cells were the most commonly stained cell type, but there were examples of olfactory neurons staining for MT (Fig 1C). Fig 1D shows immunoreactive acinar cells associated with the Bowman's glands although no evidence of immunostaining in the duct cells of the Bowman's glands was observed. Cell types were identified by their morphology, and also by their distinctive placement within the epithelium. However, since MT immunostaining in sensory neurons has not been reported before, the identity of this cell type was confirmed by co-staining with anti-olfactory marker protein antibody (Fig 1E) [7]. Furthermore, some sections were stained by double labelling, immunofluorescence histochemistry, using anti-MT and anti-OMP antisera to confirm that a subpopulation of olfactory neurons co-expressed MT (data not shown).

It is interesting to note that although examples of

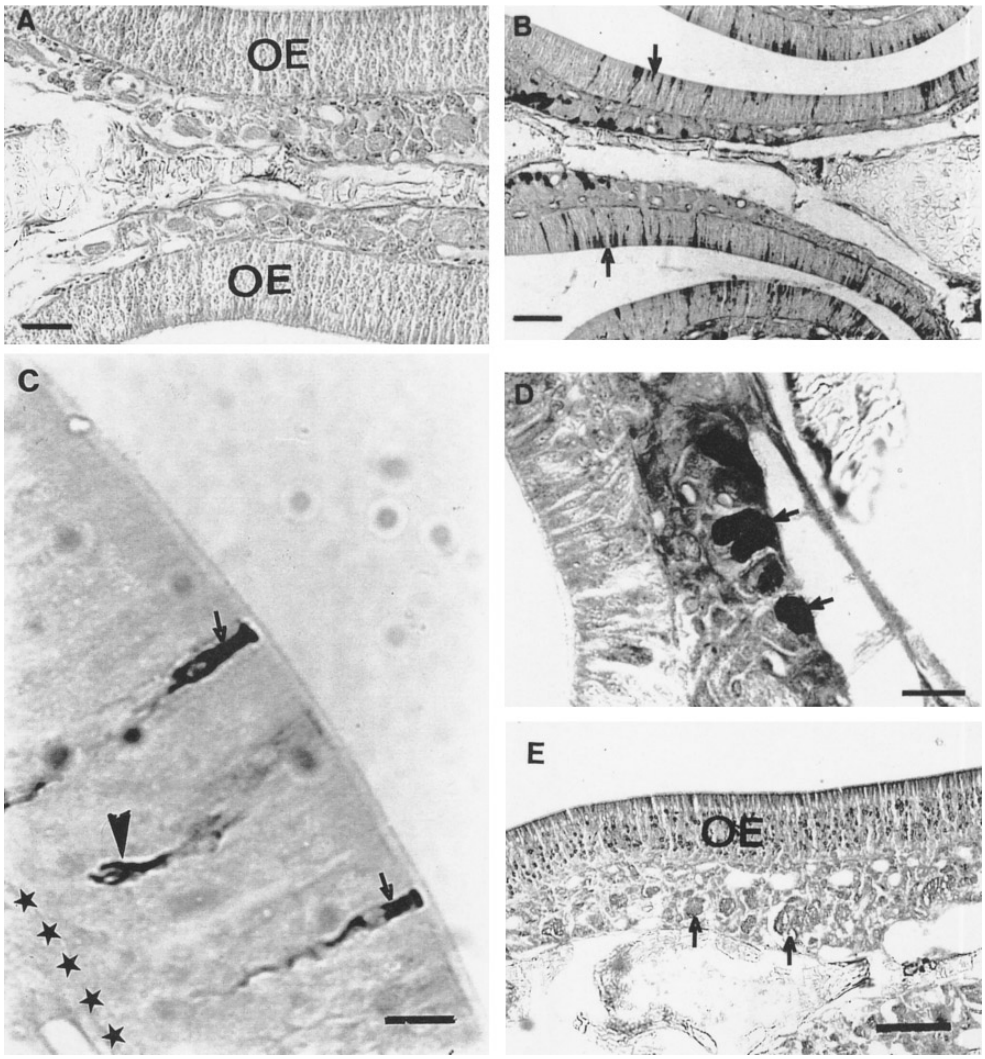

FIG. 1. Immunohistochemical staining of olfactory mucosa in wildtype mice. (A) Control section in which primary antibody against MT is omitted. No staining is present in the olfactory epithelium (OE) and the underlying lamina propria (bar = 70 μm). (B) Cells immunopositive for MT (arrows) are present in the olfactory epithelium (bar = 140 μm). (C) Most of the MT-positive cells are supporting cells (arrows) as indicated by their columnar morphology and apical location of the nuclei. A small number of MT-positive cells are olfactory neurons (arrowhead) as shown by their location in the depth of the epithelium with a dendrite extending to the surface. In this particular area, the faint positive staining above the olfactory neuron suggests there could be more neurons containing MT in the immediate vicinity. The stars indicate the boundary between epithelium and underlying lamina propria (bar = 10 μm). (D) Arrows indicate Bowman's glands showing immunopositive reaction for MT (bar = 15 μm). (E) Olfactory mucosa immunostained with anti-OMP. Positive reaction is present in olfactory neurons covering a large depth of the olfactory epithelium (OE). Their axons forming nerve bundles (arrows) also show positive reaction (bar = 100 μm).

immunoreactivity were observed for each major cell type, only a small proportion of each population contributed to the response.

MT immunoreactivity was not present over the entire olfactory epithelial surface (eg Fig 2A) as defined by OMP immunostaining (Fig 2B), but was restricted to well defined zones which were nearly identical in all animals examined. MT immunoreactivity was bilateral, with the non-expressing regions limited to the most rostral regions of the cavity roof, septum and turbinates. Fig 2C is a composite of the immunostaining seen when coronal sections from eight mice were individually traced from low-magnification video images onto a common template.

It was investigated whether the MT expression profile changes in the regenerating olfactory system. Irrigation of the nasal cavity with 175 mM $ZnSO_4$ solution produces a reproducible degeneration and exfoliation of the nasal epithelium one to two days after treatment, followed by development of a new epithelium of cuboi-

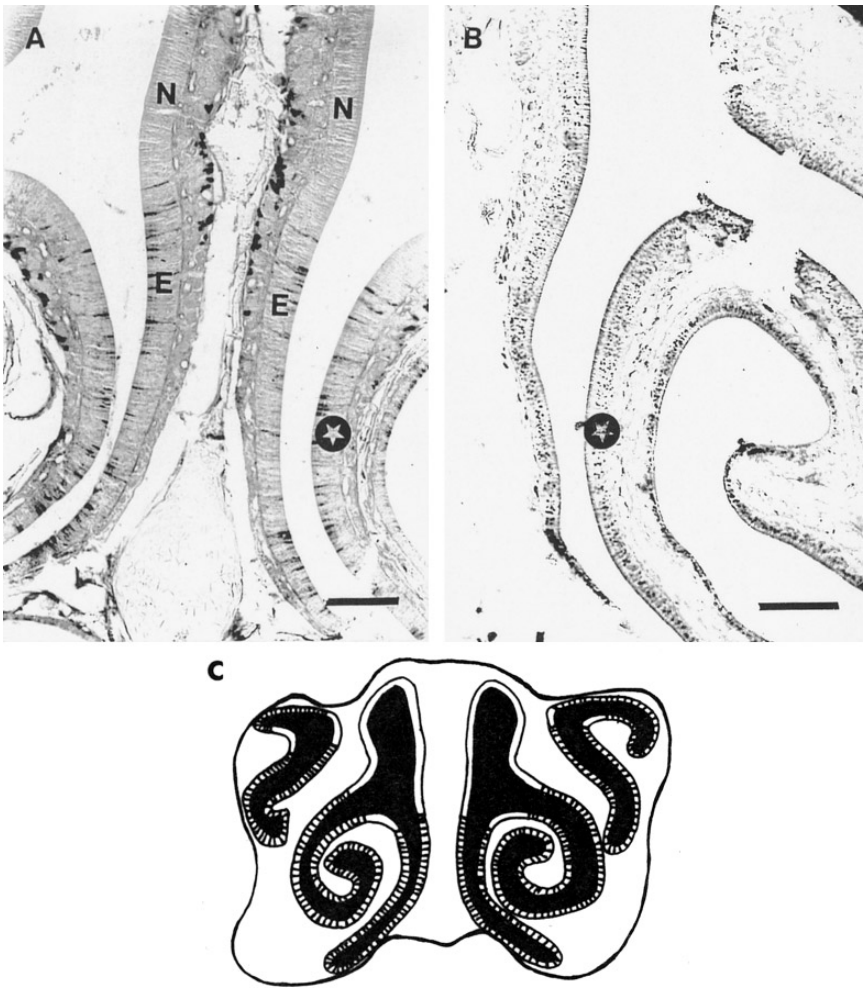

FIG. 2. (A) Anti-MT immunostaining, showing MT expressing (E) and non-expressing (N) regions. Region labeled with a star is shown in a neighbouring section (B) which has been immunostained with OMP to define the olfactory epithelium (bars in A and B = 140 μm). (C) Averaged distribution of anti-MT immunostaining over the olfactory mucosa, expressing regions indicated by cross-hatching. This diagram was derived from a composite of eight experimental animals.

dal cells, within 2-4 days. These ultimately give rise to a new, fully functional olfactory epithelium within three months [10].

Mice were treated with $ZnSO_4$ (175 mM, 3 pairs of mice for each time point: 0, 12 hr, 24 hr, 48 hr, 4 or 8 days). No obvious morphological differences were seen between wildtype and MT-deficient mice other than the latter were sometimes more prone to swelling of the lamina propria (data not shown).

$ZnSO_4$ irrigation of mice can be performed so that only one, or both, nasal cavities come into direct contact with the solution. Fig 3A shows that 12 hr after unilateral $ZnSO_4$ irrigation there was a general increase in the level of background staining in the lamina propria, in contrast to the respective regions of the untreated side. Staining was observed in the cells of the Bowman's glands on both sides. By day 4 after bilateral treatment exfoliation of the epithelium occurred, and greatly increased MT production was observed in the proliferating basal cells which have remained after exfoliation (Fig 3B).

Interestingly, when $ZnSO_4$ treatment was performed unilaterally, elevated MT immunostaining was frequently observed in undamaged cavities, first appearing about 2 days after treatment (Fig 3C). This new immunostaining was found in supporting cells and some acinar cells of the Bowman's glands and was noticeably localised to the cell nuclei. One possible explanation is that this induction of MT is produced by small amounts of zinc sulphate solution which have entered the untreated side from the irrigated olfactory chamber. However, this explanation can not account for the pattern of staining which is nuclear rather than cytoplasmic nor the time period

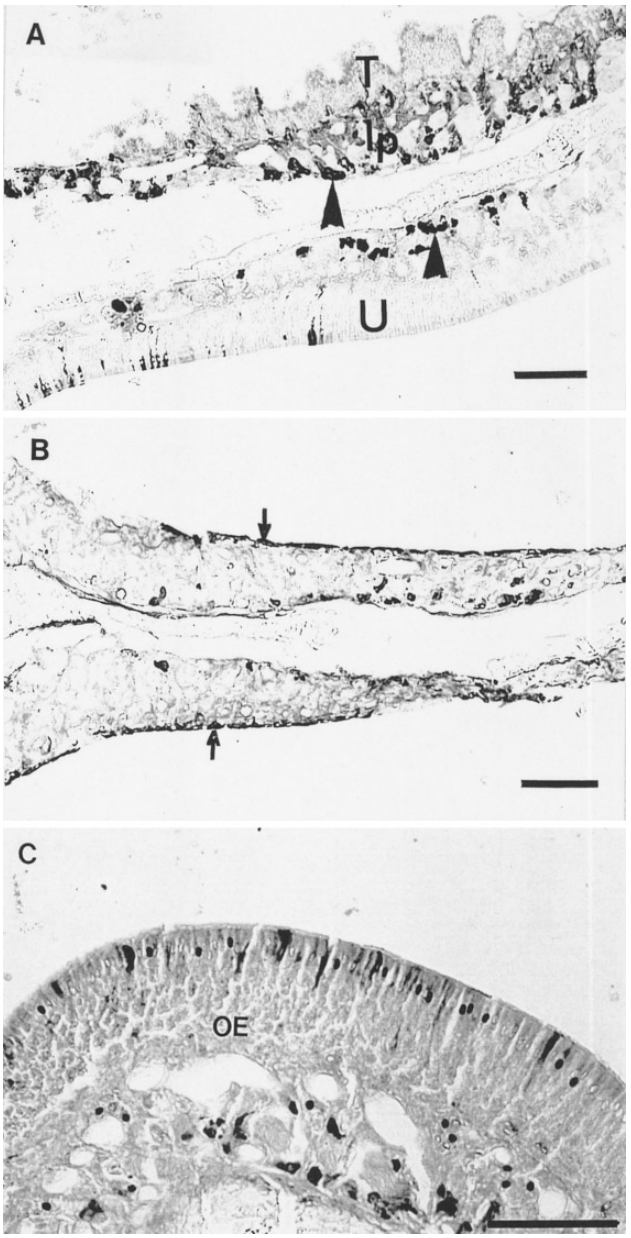

FIG. 3. Metallothionein immunoreactivity in the mouse olfactory mucosa following unilateral or bilateral $ZnSO_4$ irrigation. (A) 12 hours after unilateral irrigation, showing untreated (T) and untreated (U) mucosa on either side of the nasal septum. Bowman's glands (arrowheads) remain positive for MT on both sides while the lamina propria (lp) on the treated side shows a general increase in background staining. (B) 4 days after bilateral $ZnSO_4$ irrigation, the olfactory epithelium has sloughed off, leaving a layer of basal cells on the surface. Many of these cells show positive immunoreaction for MT (arrows). (C) Untreated side, 2 days after unilateral $ZnSO_4$ irrigation. The untreated side shows increased cellular staining for MT. The punctate pattern of staining in the apical part of the olfactory epithelium (OE) is due to the nuclear staining of supporting cells. Contrast this with the staining observed earlier at 12 hours on the treated side (e.g., A) where staining is localised to the apical cytoplasm of supporting cells (bars in A, B, and C = 100 μm).

(2 days) before which nuclear MT expression is detected. It has been observed that MT levels are increased in the remaining kidney after unilateral nephrectomy [24], and in the remnant liver after partial hepatectomy [21, 22]. In the latter case, MT becomes localised to the nucleus [21], and it has been shown using cultured hepatocytes that this process is stimulated by epidermal growth factor [22]. No nuclear localisation signal has been demonstrated yet in MT and it is not clear whether MT moves into the nucleus alone or as part of a larger complex. It is assumed that MT is required in the nucleus in its putative role in zinc or copper homeostasis, or possibly to protect nuclear components such as DNA from oxidative damage. Irrespective of its function, the nuclear localisation on the untreated side following unilateral zinc sulphate irrigation is indicative of a previously unsuspected component of the regenerative response, and may be associated with, for instance, hypertrophy or division of the expressing cells. Further work is necessary to determine whether there are more widespread changes in this region during regeneration and whether they form part of a compensatory response to loss of the remainder of the olfactory mucosa.

These data show that MT1 and MT2 are expressed widely through the mouse olfactory mucosa in a cell- and regional-specific fashion. The localisation to some olfactory neurons is particularly interesting since these isoforms of MT are not usually expressed in neurons in the CNS [1]. It is interesting that MT expression in untreated animals is confined to specific zones in the olfactory mucosa. Although the mucosa is morphologically uniform, early work indicated that specific monoclonal antibodies could demonstrate heterogeneous staining [15] in a pattern similar to that which we have seen with MT immunoreactivity. It is now believed that the mucosa can be divided into at least four zones on the basis of expression of particular sets of olfactory receptor proteins [3] We have found that MT expression appears to be found in the K4/K7 and the OMP-only zones, and not in zone K18, as defined by Ressler et al [18]. This co-localisation may have functional significance. Another example of a protein which is expressed in a zonal fashion includes an isoform of phenol sulfotransferase which is confined to the dorso-medial portion of the nasal cavity [14], the region which is exposed to the highest levels of inhaled compounds. Thus, it is possible that the zonal organisation of the epithelium reflects differing functional properties, possibly relating to odorant clearance or toxicity. MT is induced by many xenobiotic compounds in other organs such as the liver and it is possible that its expression in specific zones of the olfactory mucosa suggests a protective role for this

protein. Before interacting with the chemosensitive membrane of olfactory neurons, molecules in the nasal cavity must traverse a protective layer of olfactory mucous secreted by the Bowman's glands and supporting cells. The mucus layer functions as a physical and chemical barrier and furthermore it has been shown that organic esters absorbed by the olfactory epithelium are hydrolyzed by carboxyesterases in the supporting cells and Bowman's glands (Bogdanffy and Frame, 1994). Hence, it is consistent that increased MT expression is found particularly in these two cell types.

It is also intriguing to note that MT expression in the untreated mouse does not appear uniformly in all cells of a specific type, but is restricted to a subset of individual cells within an expressing zone. It has been suggested previously that dividing cells express higher levels of MT than quiescent cells [22], however the possibility that the MT expressing cells in this study were at specific stages of the cell cycle seems unlikely since preliminary experiments suggested there is no obvious correlation between MT and Proliferating Cell Nuclear Antigen (PCNA) immunostaining (data not shown). PCNA is a marker of cells in S-phase [16]. This should be more rigorously tested however, possibly by MT/PCNA immunofluorescent double labelling. However, during regeneration of the olfactory epithelium following $ZnSO_4$ treatment, MT expression was most pronounced when the epithelium was one or two cells thick and the basal cells were proliferating to reconstitute the epithelium. This work also suggests that MT 1 and 2 isoforms are not obligatory for the initial stages of regeneration of the olfactory mucosa, since the MT-deficient mice did not show obvious morphological differences in this regard compared to wildtype mice following $ZnSO_4$ irrigation.

In summary, MT immunoreactivity, attributable to MT1 and MT2 isoforms has been localised to subpopulations of specific cell types within the mouse olfactory mucosa. Expressing cells are organised into distinct regions of the epithelium. MT1/2 expression is not a requirement for regeneration of the olfactory mucosa following $ZnSO_4$ exfoliation although the protein was detected in the proliferating basal cells. Nuclear MT was observed in supporting cells and acinar cells of Bowman's glands on the untreated chamber following unilateral $ZnSO_4$ treatment.

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