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An in vivo study of utricular hair cell degeneration and regeneration following aminoglycoside induced damage of the mammalian inner ear

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A thesis presented for the degree of Doctor in Medicine

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September 2005

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Abbreviations

HC	Hair Cell
SC	Supporting Cell
LM	Light Microscopy
TEM	Transmission Electron Microscopy
IHB	Immature Hair Bundle

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There are many pioneers in the field of investigating hair cell regeneration in fish, amphibians, reptiles, birds and mammals.

‘ Their imminence was eminent before our eminence was even imminent’

Hugh Laurie

Declaration

I, Peter Walshe declare:

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Abstract

The anatomy of the mammalian ear is similar between the different subspecies. This applies to the structure of the utricle in humans and in guinea pigs. Hence the choice of the guinea pig utricle allows any findings pertaining to the physiology, development and anatomy to be applied to the human inner ear with a high degree of confidence.

It has been known for some time now that the mammalian inner ear has a certain limited capacity to regenerate after damage. It is not yet known however where the regenerative attempt arises at a cellular level.

The middle layer of the utricle is the macula –a sensory organ of balance. It contains hair cells – the impulse generators and supporting cells – their associated metabolic support cells.

In this study the maculae of thirty five guinea pigs were damaged by transtympanic membrane injection with gentamicin. The opposite ears served as controls.

The maculae were harvested at 1 (ten animals), 2 (ten animals) and 4 weeks (ten animals) post gentamicin damage and analysed at light microscopy and transmission electron microscopy for changes in hair cell and supporting cell numbers.

A further 5 animals were treated with gentamicin and also intraperitoneal taurine as a potential cytoprotectant. These animals were harvested at 2 weeks and the maculae examined at light microscopy and transmission electron microscopy.

Results showed evidence to support hair cell repair as a method of regeneration, that the migration of supporting cell nuclei in response to gentamicin induced inner hair cell death appears to be nothing more than artifactual, that taurine does not increase or

decrease the transmission electron microscopy evidence for potentiating hair cell loss in gentamicin induced toxicity and that direct transdifferentiation of supporting cells appears to be a potential origin to hair cell regeneration in mammals.

Future studies involving growth factors, gene manipulation, heat shock protein, capsase inhibitors and stem cells may yield further evidence to support and indeed manipulate mammalian inner ear regeneration.

Anatomy and ultrastructure of the mammalian utricle

Gross Anatomy

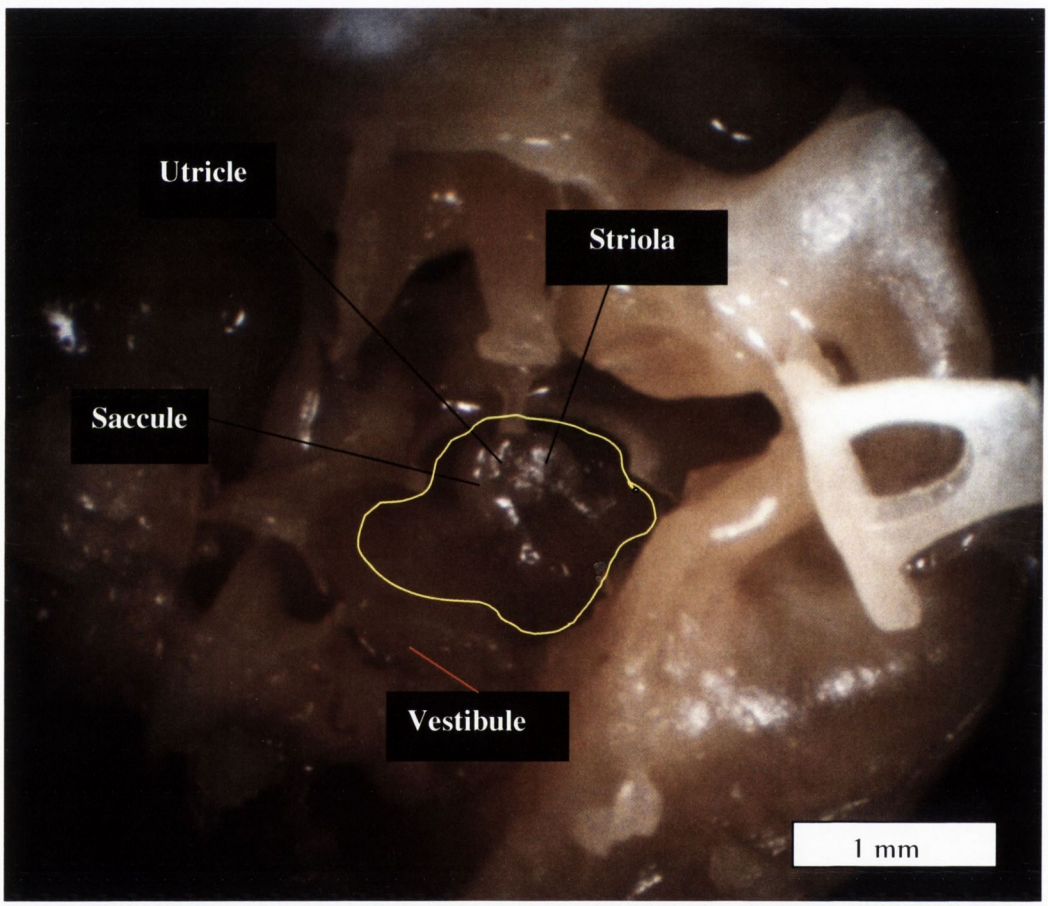
The anatomy of the mammalian ear is similar between the different subspecies. This applies to the structure of the utricle in humans and in guinea pigs. Hence the choice of the guinea pig utricle allows any findings pertaining to the physiology, development and anatomy to be applied to the human inner ear with a high degree of confidence.

The utricle is a flat kidney shaped sensory organ which is found in the posterosuperior part of the vestibule, a bony chamber in the inner ear. Its axis lies in the horizontal plane in the anatomical position, an arrangement which permits it to detect positional changes in the horizontal plane. The surface area is appoximately 0.54 mm^2 in guinea pigs. It is approximately 3.6 MM^2 in humans.

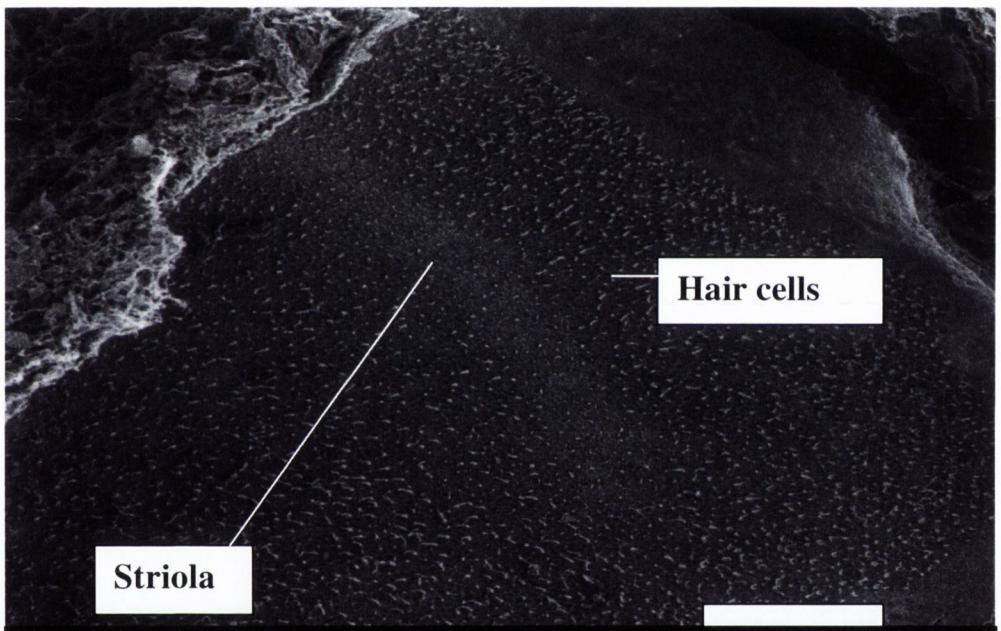
It is responsible for relaying data about the position of the body in space to the brain and has a role in maintaining balance via the vestibulospinal tract and maintaining the control of eye movements via the vestibulo – ocular pathways.

Like all sensory organs the utricle's function is dependant on specialised sensory cells which function as impulse generators. These cells are called hair cells and are mechanoreceptors which convert movement generated distortions of their structure into electrical impulses.

The density of the hair cells in the comma shaped central striolar region is significantly less than the non striolar region.



Dissected guinea pig ear showing the vestibule and its contents



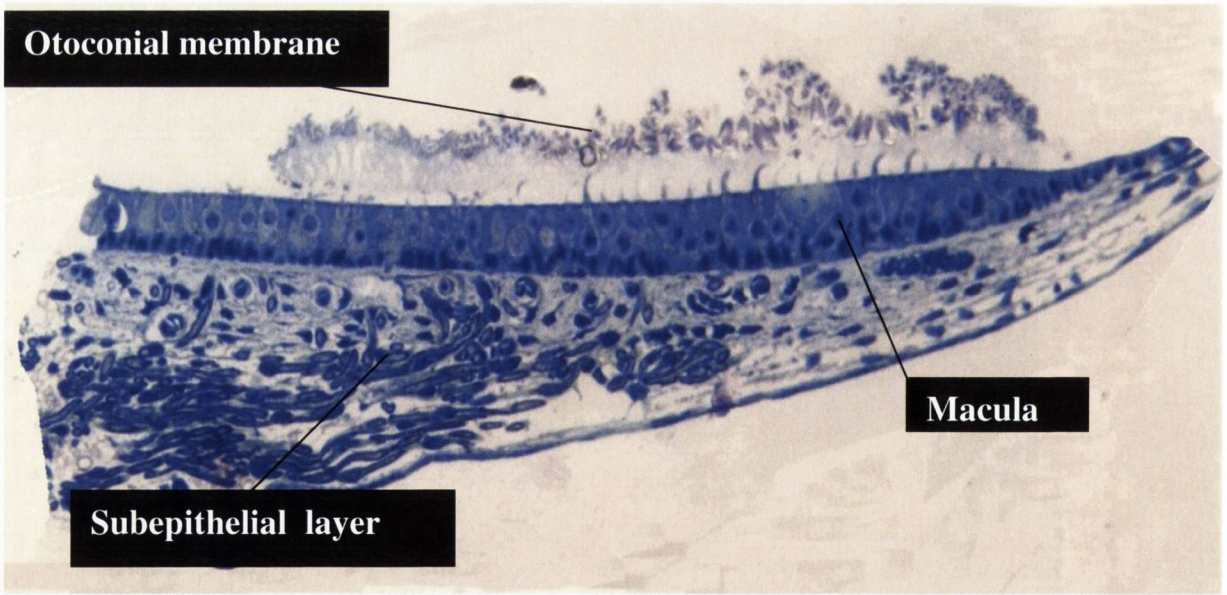
Scanning electron micrograph of the guinea pig utricle. The scale bar indicates 100 μm. Reproduced with kind permission from Trinity College Dublin.

The utricle is made up of three distinct layers.

The most superficial layer is the **otoconial membrane**.

The deepest layer, the subepithelial layer or **neurovascular layer** contains nerves and blood vessels.

In between is the **epithelial layer** or macula.



Cross section of a guinea pig utricle stained with Toludine blue (LM x 40)

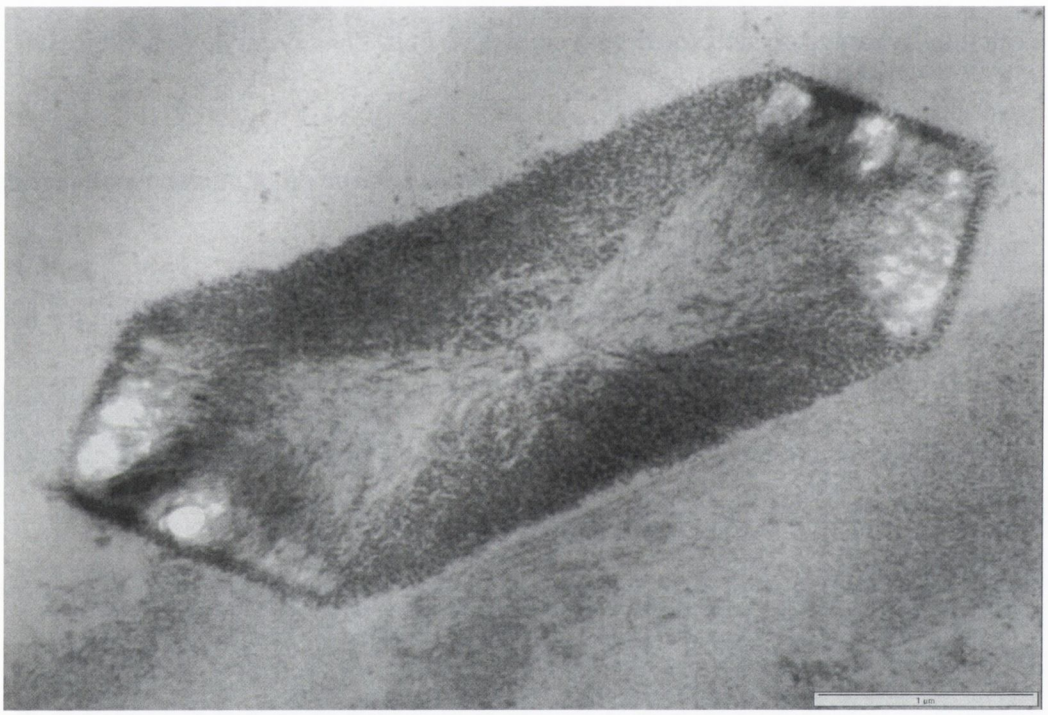
The otoconial membrane

The most superficial layer of the macula is a gelatinous layer (the otoconial membrane). This consists of a gelatinous membrane which has a crystal layer of calcium carbonate crystals (otoconia) embedded in it. The crystal layer is thinnest over the striola (Lindeman 1973).

The gelatinous portion of the otoconial membrane is made up of mucopolysaccharides and collagen. It is least dense in the lower layers.

The supporting cells make contact with the membrane at their apical ends but the hair cells project into pre - formed canals in the membrane which act as channels. The channels appear to be in contact with the hair cells longest stereocillia and kinocillium in the periphery of the utricle (Engstrom et al 1962) but in the striola there appears to be a free space between the stereocillia / kinocillium and the channel walls. It is believed that the otoconial membrane is secreted by the supporting cells (Kawomata S, Igarashi Y. 1993).

The otoconia are of three shapes – pyramidal, rounded and a mixture of the two. They measure 0.5 – 3.0 μm in the guinea pig and 1 – 20 μm in humans (Carlstrom et al. 1953). Each otoconium has a compact central core meshwork of fillaments and a composite outer shell of crystalline aggregates (Lins et al 2000).



TEM of calcium carbonate crystal which constitutes an otolith.
Mag. X 30,000

The neurovascular layer (the deepest layer of the utricle)

The blood vessels in this layer originate from the superior vestibular artery and nerve (macula of the utricle) and the inferior vestibular artery and nerve (macula of the saccule) (Mazzoni A 1990).

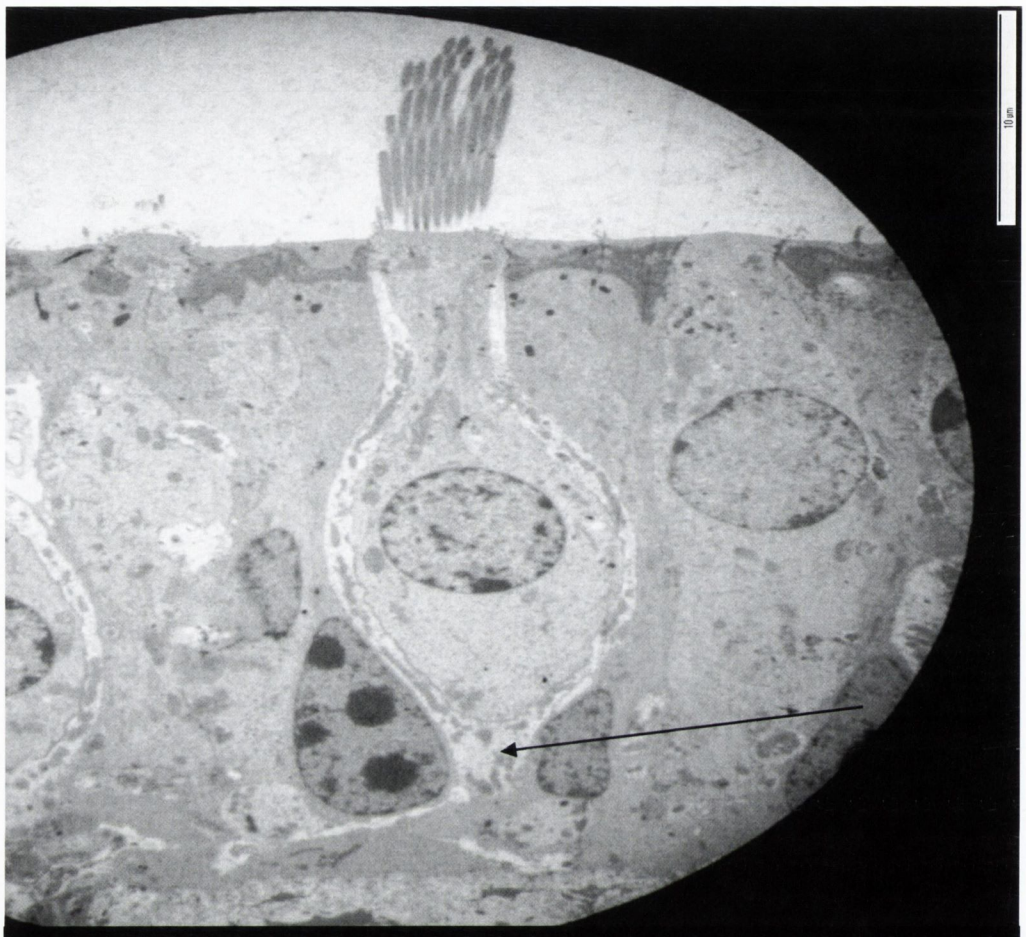
Vascular arrangement of the macula (and cristae of the semicircular canals) is of a three layered architectural arrangement with a subepithelial layer, a middle dense layer and a deep loose layer.

There are about 1,700 innervating nerve fibres in the neurovascular layer, mostly afferent. They arrive via the utricular branch of the superior division of the vestibular nerve (Honrubia et al. 1987). Afferent neurons arrive via bipolar cells in Scarpas ganglion whereas the efferent nerves travel to the vestibular nuclei. Fibres are both myelinated and unmyelinated but myelination is lost as the neurons penetrate the basement membrane (Honrubia et al .1987).

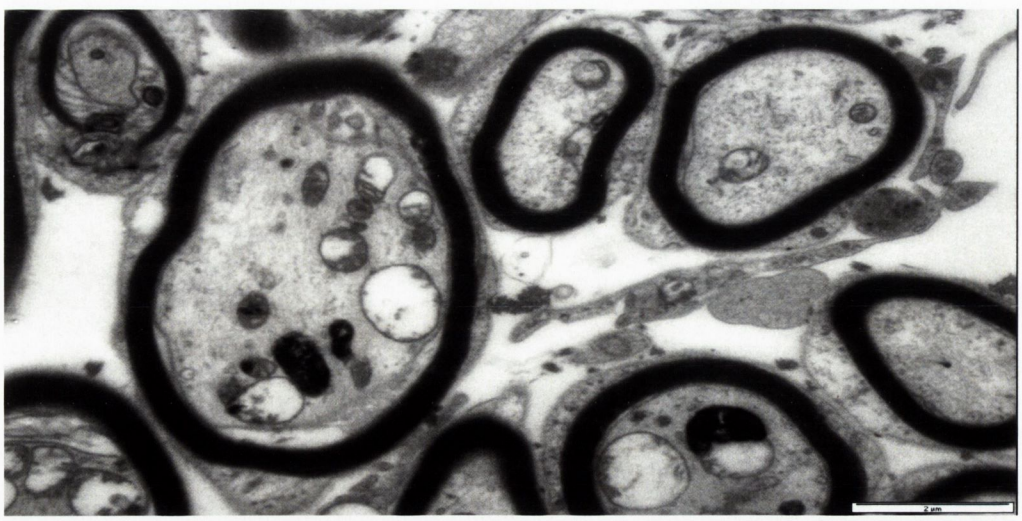
The medium and larger diameter fibre nerves supply the type I Hair cells particularly around the striola. The afferent innervation of the type II hair cells is via medium and small diameter fibres. The differences between type 1 and type 2 Hair Cells are discussed on page 16.

Judging by the total number of hair cells in the utricle and the total number of nerve fibres arriving at the utricle, it has been estimated that each nerve fibre supplies between 4 – 8 hair cells. The ratio of hair cells : axons does however differ in each end organ in the inner ear. Recent evidence suggests that the utricle has the highest ratio (23:1) (Mathiesen and Popper 1987).

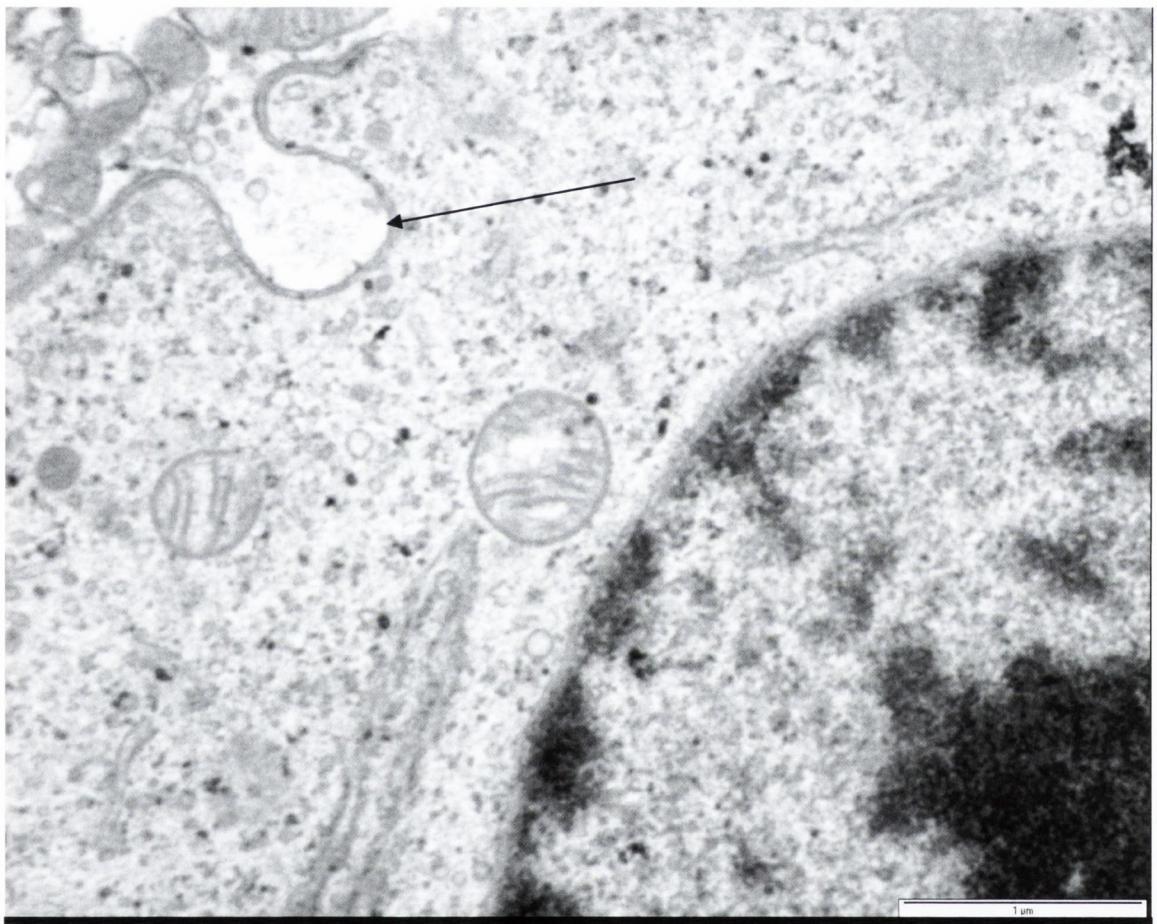
The efferent fibres form a network basally in the epithelium and give off numerous collaterals particularly to type II cells .



TEM showing entry of a nerve from the deep layer to form a chalice around this type I hair cell (X 3000)



TEM showing myelinated nerves of the deep layer of the utricle in cross section (X 15,000)



TEM showing a nerve vesicle at the basal end of this hair cell after the nerve has penetrated the basement membrane (X 20,000)

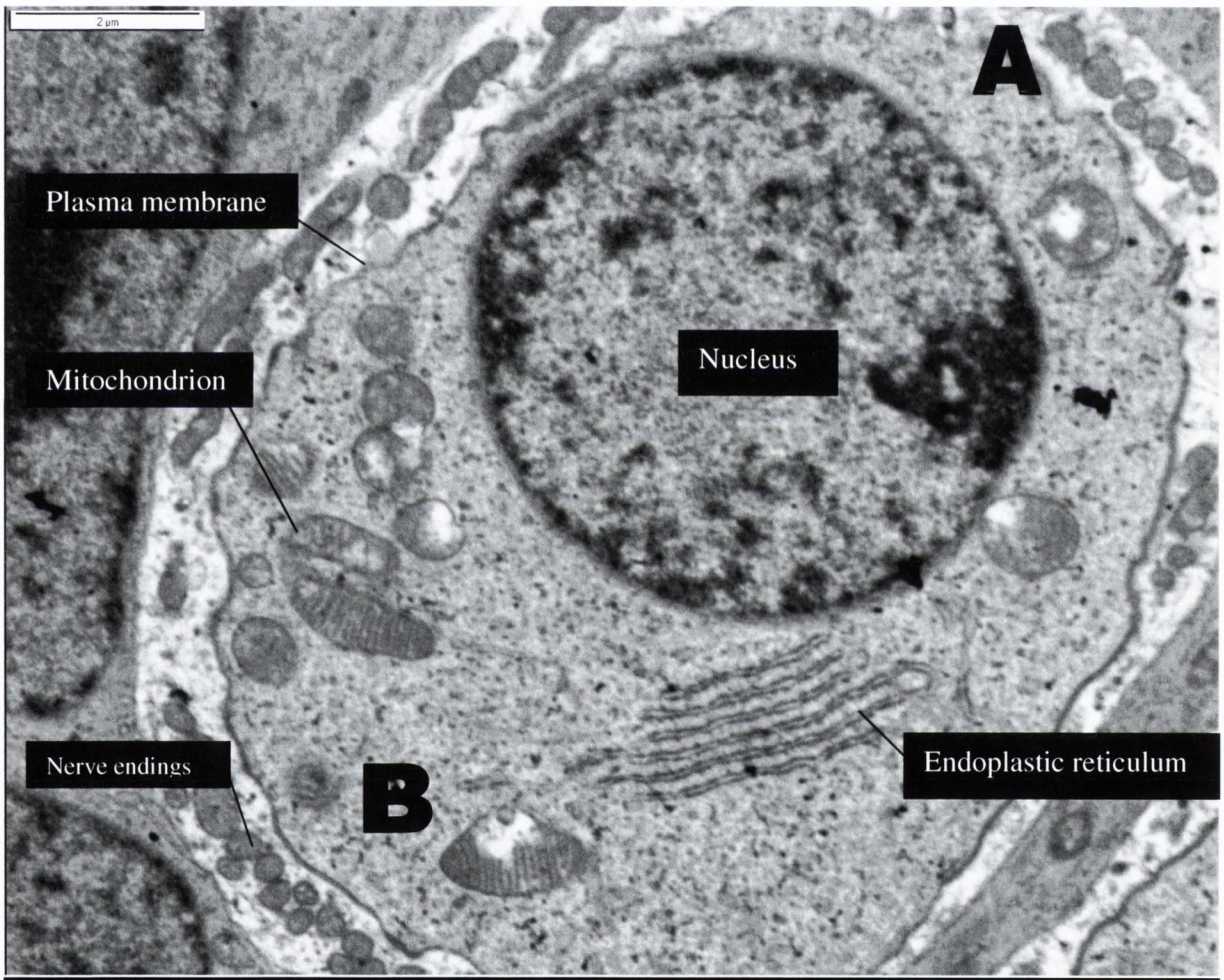
The Macula

The middle layer or epithelial layer of the utricle and saccule is called the macula. It is found in the lower part of the lateral wall of the utricle. The plane of the macula in the utricle is oriented perpendicularly to the plane of the macula of the saccule in the vestibule. It consists of sensory hair cells and supporting cells. There are approximately 9,260 hair cells in the guinea pig utricle and 33, 100 in the human utricle (Rosenhall 1972).

There are numerous nerve connections between the supporting cells and the hair cells (Lindeman 1973). The supporting cells are thus intricately related to their adjacent hair cells.

The apical surfaces of the hair cells and the supporting cells form a network at the luminal surface called the reticular lamina. This forms a barrier between the perilymph (which surrounds the cell bodies) and endolymph (which is at the cell apex). The apical surface area of the hair cells in the striolar region is much larger than that of hair cells in the non striolar region. It is not surprising therefore that the density of hair cells is significantly lower in the striolar region than in the periphery of the utricle (Lindeman 1973).

Hair cells



Transmission electron micrograph (cross section) of a hair cell showing the ultrastructure and organelles. A indicates the apical end, B the basal end. (Mag. X 6000)

The sensory cells are of two types – type I and II, named according to the shape of their cell body and innervation pattern (Wersall 1956). The function and role of each hair cell type is unknown however there are twice as many type I cells as type II cells in the striolar region whereas the ratio in the periphery is approximately 50 : 50.

Type I hair cells are shaped like a flask and each cell body is surrounded by a large goblet shaped nerve terminal. These nerve terminals can surround many cell bodies of different hair cells. Type II hair cells have cylindrical shaped cell bodies and a cluster of nerve terminals at the basal region of the cell body.

The afferent neurons arising from type I cells are larger than the type II cells.

The efferent nerve fibres to the type I cells end on the nerve chalice whereas those to the type II cells end on the cell bodies of the hair cells.

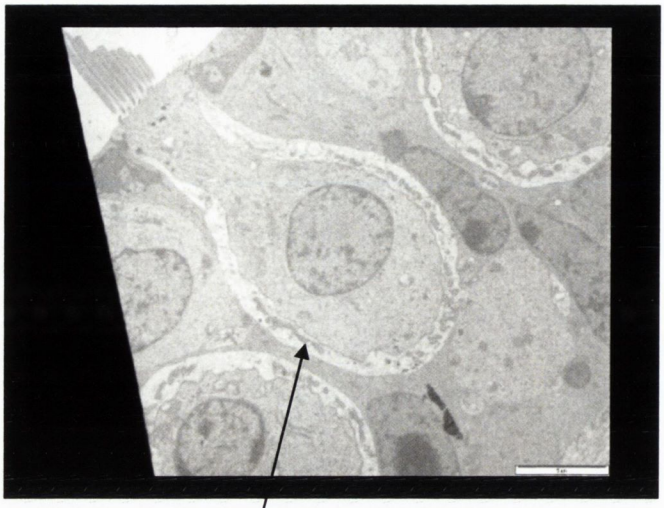
Each hair cell is embedded in an epithelium made up of supporting (sustentacular) cells. The basal end of the hair cell is in close contact with the afferent neurons which run from it to the vestibulocochlear nerve.

Approximately 50 - 110 hairs project from the apical end of each hair cell (Lindeman 1973). The single kinocillium present in each utricle hair cell apex is the only true cillium having the classical 9 x 2 arrangement of microtubules in its ultrastructure. It and only it has a basal body associated with it. The kinocillium is located at the edge of the mature hair cell. The dynenin arms and central microtubules are absent in guinea pig kinocillia (Kikuchi et al 1989).

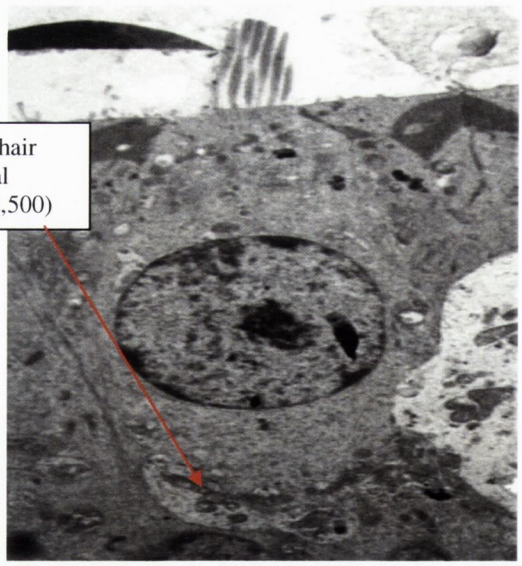
The stereocillia which surround the kinocillium do not have a typical cilliary arrangement. They are therefore pseudocillia.

Stereocillia are modified microvilli and possess actin filaments cross linked by fimbrin. This causes them to be relatively rigid. They contain rootlets and a narrow base known as an ankle. The fact that stereocillia are linked by cross links to their

neighbours and to the kinocillium (Bagger – Sjoback and Takumida 1988) means that the whole hair bundle can move as a unit upon deflection by a movement in the vestibule (or indeed a travelling sound wave for the hair cells in the cochlea). Further links between the tips of smaller stereocillia and the larger adjacent stereocillia have been described (Osbourne et al 1984).



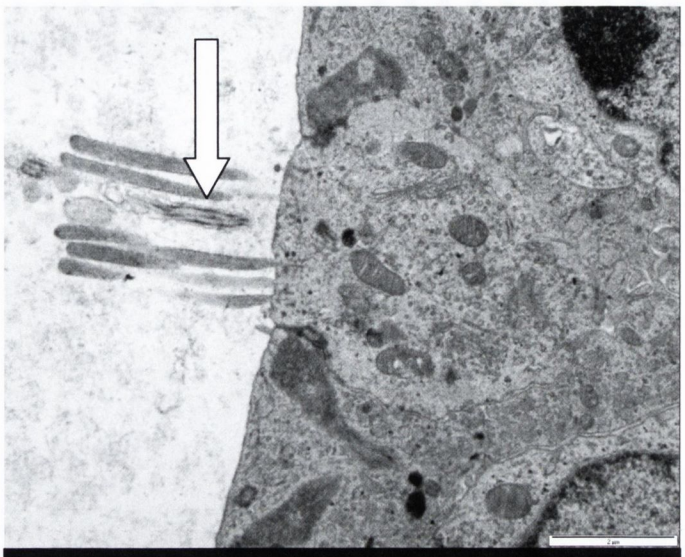
Type 1 hair cell with nerve terminal highlighted (Mag. X 2,500)



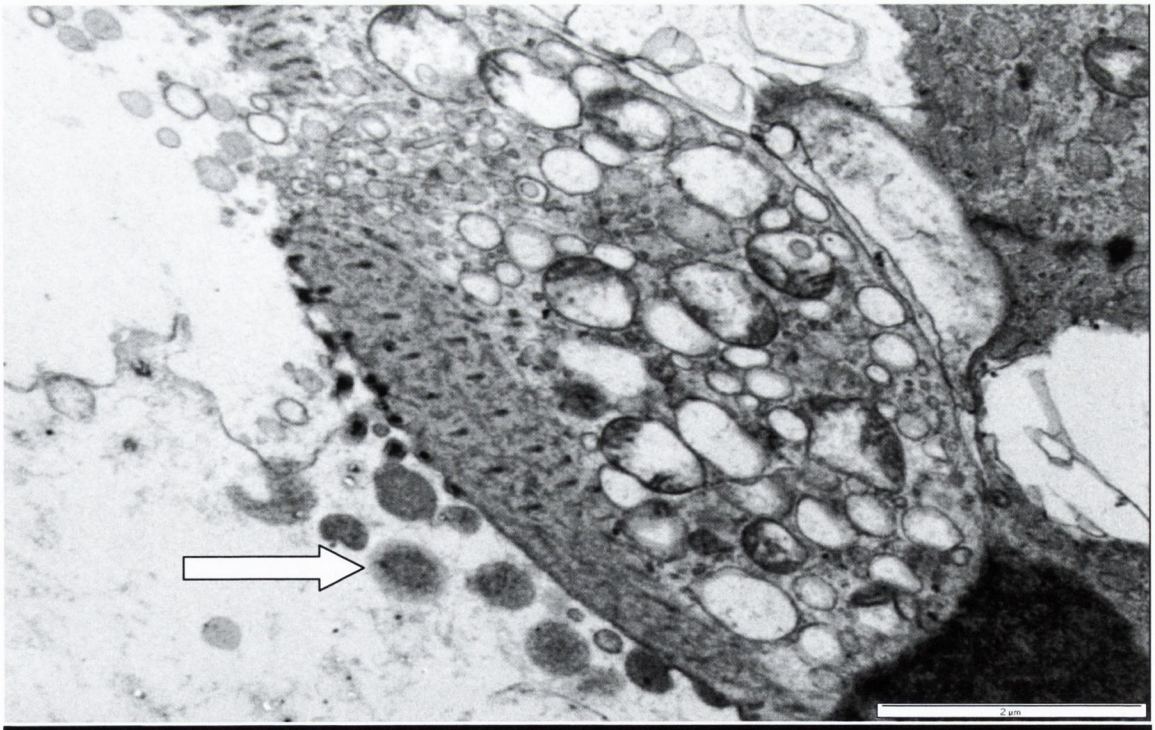
Type 2 (regenerating) hair cell with nerve terminal highlighted. (Mag. X 2,500)



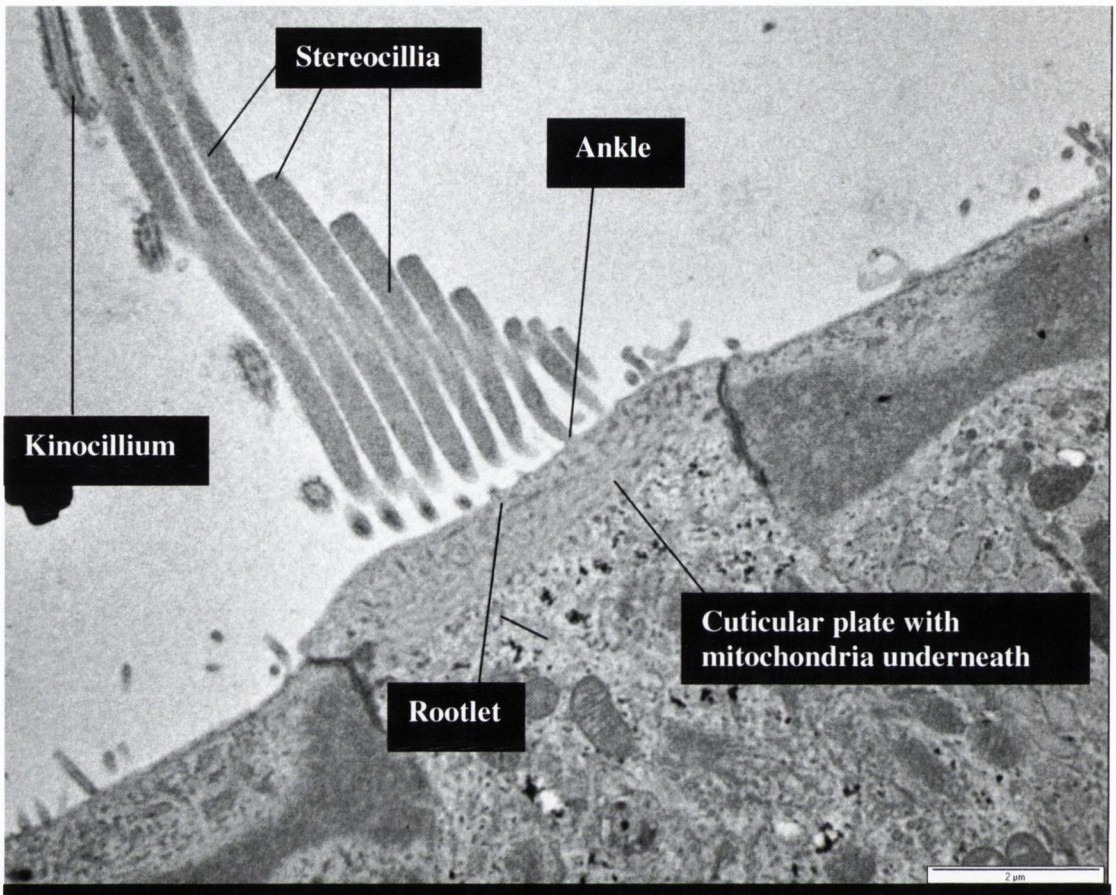
Basal body of the kinocillium. (Mag X 10, 000)



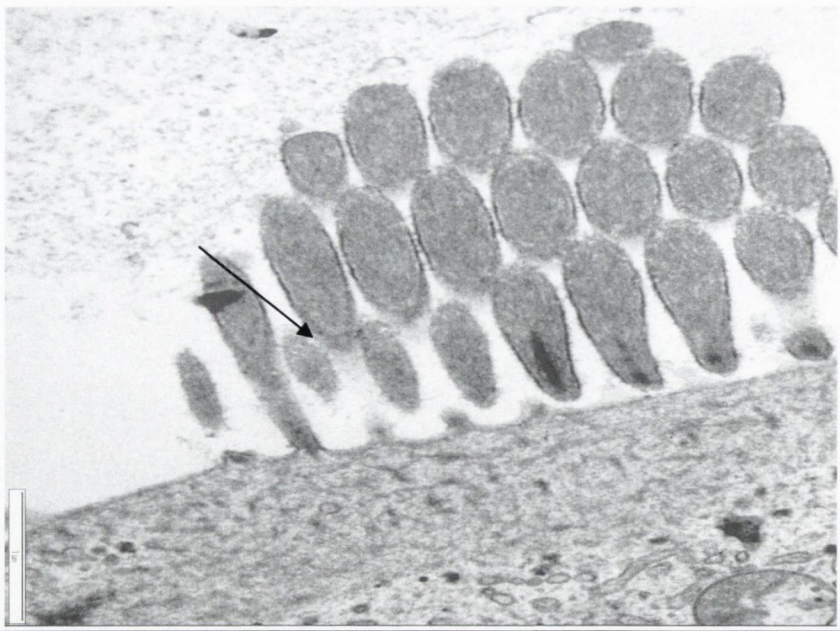
The microtubules of only the kinocillium is seen on this TEM as the stereocillia do not possess them (Mag. X 4000)



Note in the kinocillium that neither the dynenin arms or the central microtubules are present. This is a feature of the guinea pig inner ear and is not found in common with humans (TEM x 4,000)



TEM of a type I hair cell bundle (X 5000)



TEM showing a possible cross link between the apex of the shorter stereocillium and its taller neighbour. The cross links are broken down by the embedding process making identification difficult. (X 10, 000)

Polarity

As the kinocillium is eccentrically placed (at the edge of the hair cell apex) it is morphologically polarised. The striola separates hair cells of opposite polarity.

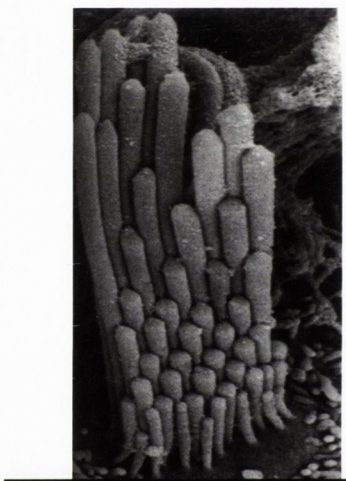
The striola versus the periphery with regard to hair bundles on hair cells

The hair bundles on striolar hair cells (S - type hair bundles) are shorter than those in the periphery (P-type hair bundles) (Kessel and Kardon 1979).

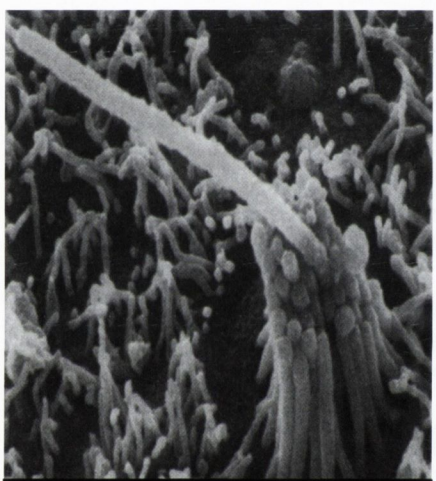
To further complicate matters the hair bundles are divided and classified according to the relative heights of the kinocillium and stereocillia.

Type I bundles have a kinocillium and tallest steroecillium of approximately equal heights. The stereocillia are club-like.

Type II bundles have slender steroecillia and a very tall kinocillium.



Type I bundle seen at scanning electron microscopy



Type II bundle seen at scanning electron microscopy

Reproduced with kind permission from Trinity College Dublin

Type I bundles are presumed to arise from type I hair cells and type II bundles from type II hair cells (Lim 1977). This was confirmed in this study.

Physiological responses of the hair cells

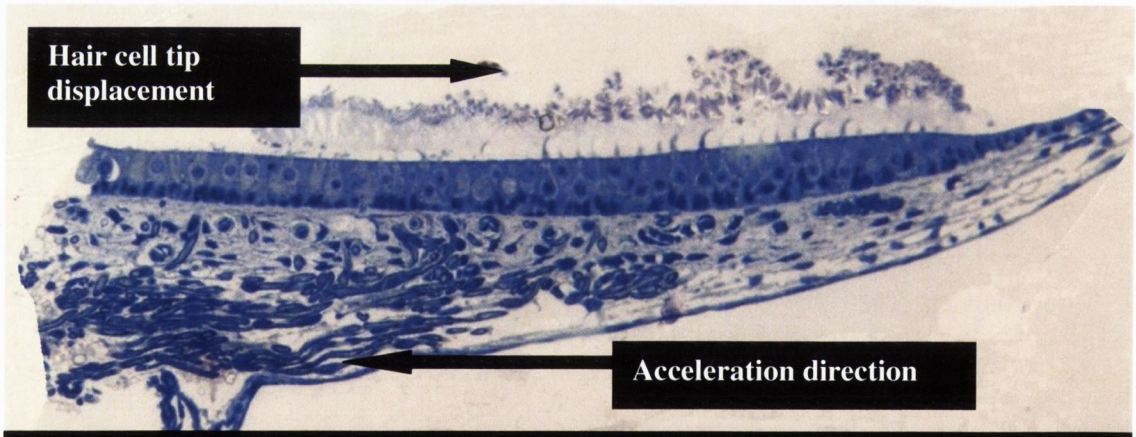
The resting apical membrane potential of the hair cells is about - 60 MV. When an animals head is accelerated in the horizontal plane, the stereocillia are displaced towards the kinocillium and the membrane potential difference decreases. When pushed in the opposite direction the potential difference increases and the cell becomes hyperpolarized (Lowenstein and Wersall 1959; Russell et al 1986). Displacement perpendicular to this axis gives no change in polarity, whereas displacement at an angle gives a response proportional to the angle of displacement with respect to the kinocillium. The response is most sensitive when activated from rest and as little as 100nm displacement (1 degree of angular displacement) of the stereocillia will elicit a response (Hackney and Furness 1995).

The genesis of this action potential has its basis in ion flux across the membrane. When the stereocillia are displaced towards the kinocillium the apical ion permeability increases, potassium enters the cell and opens voltage gated calcium channels. This allows calcium influx and subsequently the hair cell becomes depolarised, thereby setting up a genertor potential in the afferent neuron supplying the cell.

Responses to movement

The otoliths in the vestibule of the inner ear have a certain inertia and are therefore displaced in a given direction more slowly than the bone around them. This causes the tips of the hair cell processes to bend. When linear speed is constant the otoliths move at the same pace as the body and therefore the hair cell stereocillia spring upright again. When linear deceleration occurs the inertia of the otoliths causes them to again displace the hair cell processes, this time in the opposite direction.

Maculae discharge tonically due to gravity also.



Relative inertia of the otoliths temporarily displaces the tips of the hair cells in the opposite direction to the acceleration of the head. The black arrow represents the direction in which the head has accelerated and the white arrow the direction of displacement of the hair cell tips. The opposite effect is noted when the head decelerates

Embryology of the mammalian labyrinth

The immature hair cell bundle is a feature of hair cells which are attempting to regenerate after insult in the mammalian inner ear. Their name derives from the observation that they resemble the immature hair cells found during normal mammalian inner ear development.

As regeneration of these hair cells in the inner ear requires a development of hair cells from a progenitor source, the understanding of the normal development of the inner ear is helpful in identifying a possible cellular source of regenerating hair cells in the utricle. This understanding will also allow us to identify immature hair cell bundles and differentiate them from normal undamaged hair cells.

Sequence of events

The gross development of the vestibular labyrinth follows three stages. Firstly a membranous labyrinth forms. Secondly this becomes encased in bone and thirdly spaces form in the bone.

In humans, the vestibule like the rest of the inner ear forms when at about 22 days of embryonic development a thickening of ectoderm from adjacent to the neural groove (the otic placode) sinks below the surface into the mesoderm to form the otic pit and subsequently the otocyst.

Neural crest cells form the vestibular ganglia. This otocyst forms the membranous labyrinth by 25 weeks.

The mammalian inner ear develops in a similar fashion albeit at a different rate depending on the gestation time of the different species. For obvious reasons the human vestibular system development has not been as fully and thoroughly documented as other species. Fortunately the mouse has been examined in detail and serves as a good model to draw comparisons with humans.

In the mouse at about day 12 or 23 two sacs appear as outpouchings from the membranous labyrinth - the endolymphatic sac and the cochlear duct. Subsequently the labyrinth forms the pars superior and the pars inferior. The former forms the utricle and the semi-circular canals, the latter the saccule and the cochlea. The utricle and the saccule have appeared by day 13 to 14. The superior semicircular canal duct appears from the dorsal wall of the otocyst between day 12 to 13 followed by the posterior semicircular canal which has formed by the end of day 13. The lateral semicircular canal duct and cristae appear by day 14.

In both humans and mice the number of hair cells in the inner ear sensory epithelia

declines with age, indicating cell death. However since reports demonstrated the ability of the vestibular sensory epithelia to regenerate after injury (Forge et al. 1993, 1998; Kuntz and Oesterle 1998; Li and Forge 1997; Rubel et al. 1995), much interest in the area of mammalian inner ear regeneration has developed.

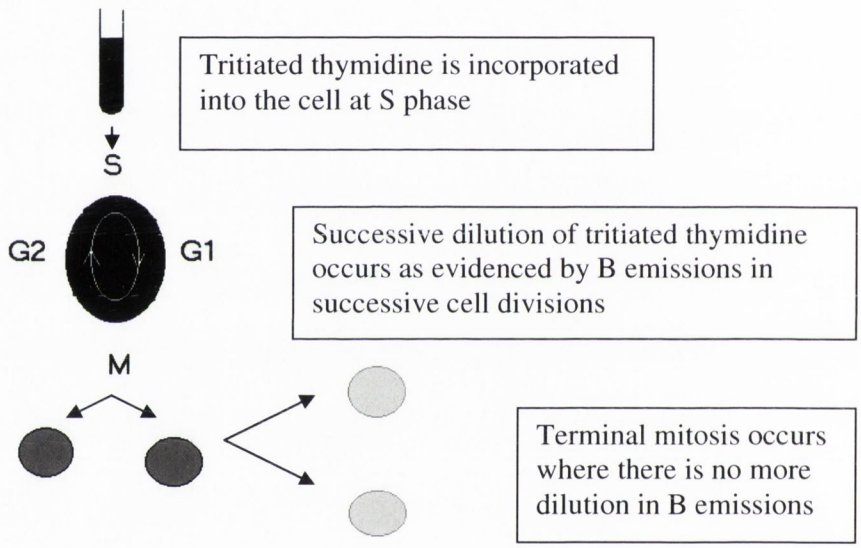
A continuous hair cell turnover in the vestibular epithelia has not previously been demonstrated in mature mammals.

Bats are the only true flying mammals, and they are known to live to a higher age than other mammals of equal size. The young are fully developed and able to fly at the age of 2 months, and thus the vestibular organs are thought to be differentiated at that age. Consequently, long-lived mammals such as bats might compensate for the loss of hair cells by producing new hair cells in their postembryonic life. It has been shown that the utricular macula of adult Daubenton's bats (more than 6 months old) contains innervated immature hair cells as well as apoptotic hair cells, which strongly indicates a continuous turnover of hair cells, as previously demonstrated in birds (Kirkegaard M, Jørgensen JM 2000).

The concept of Terminal mitosis

This serves as an index of when a fixed non dividing cell population is formed.

The timing of terminal mitosis can be established experimentally by using tritiated thymidine as shown below.



Terminal mitosis takes place for the inner ear hair cells and supporting cells in utero in mammals (Ruben ' 67) (Sans and Chat '82).

Terminal mitosis occurs in the hair cells and supporting cells of the mouse at day 13 – 15 (cochlea) and day 14 – 18 (Vestibule). Macular cells terminate before those of the cristae (Ruben '67) and this occurs from the striola to the periphery. In the cristae it occurs supero-inferiorly (Sans and Chat ' 82).

Differentiation of hair cells and stereocillia

In the human fetus it appears that synaptogenesis begins before ciliogenesis (week 10 of gestation versus week 12) at least with respect to the development of the cochlea. In the inner hair cell (IHC), an adult - like stage is seen around week 15 for synapses, but not before week 22 for stereocilia. In the outer hair cell (OHC), both synapses and stereocilia are not yet fully mature at week 22. Classic gradients of maturation are found: a base-to-apex gradient, and an inner hair cell (IHC) to outer hair cell (OHC) gradient. By comparing these results with the anatomical and functional data on cochlear development in laboratory mammals, the onset of cochlear function in the human fetus can be estimated to occur around week 18. The completion of cochlear maturation based upon the same anatomical criteria should occur about 10 weeks later.

Cytodifferentiation occurs from the centre to the periphery in the macula in the mouse (Li ' 78) (Sans and Chat '82) (Lim and Anniko ' 85). This process has been extensively studied in mice and from these studies we have acquired most of our knowledge on cytodifferentiation of the mammalian vestibular apparatus.

The cells which form the maculae form two distinct layers in the sensory epithelium. Tall columnar cells that have rounded nuclei travel to the luminal surface to become hair cells. The larger of these cells have immature hair bundles. The cuticular plate has not developed and the kinocillium is peripheral. The cytoplasm has large numbers of mitochondria, clusters of ribosomes and irregular endoplasmic reticulum and microtubules. In the early stages all hair cells have no synapses and are of a type 2 morphology (Sans and Chat ' 82) (Kawomata and Igarashi '93).

In the mouse at day 17 the regularly arranged hair bundles are visible and neurones

have begun to form synapses. These become complete by day 19. Differentiation of the type II hair cells to type 1 begins to occur by day 18 although expression of a voltage gated channels which are activated at resting potential and are specific to type I cells is observed between day 4 –8. This indicates that electrophysiological differentiation begins at this stage. Indeed when mouse utricles from day 0 to 28 were examined at transmission electron microscopy and compared to electrophisiological testing of utricles harvested at the same times the electrophysiological and ultrastructural differentiation followed different time courses. By day 28 the two had converged. Interestingly data from denervated cultures showed that neither morphological or electrophysiological differentiation depended on ongoing innervation. Hence the utricular hair cells undergo primed ultrastructural differentiation prior to synapse development. (Flock et al '73) (Van Der Water '76), (Rusch et al 1978).

Indeed the nerve chalice arrangement of type 1 hair cells is incomplete at birth and the efferent system less well developed at birth than at full maturity. Vestibular efferent network changes in the rat utricle have been examined during a 17 day period of microgravity. This was achieved by exposing newborn rats to the atmosphere of space aboard a space shuttle flight from 8 days old to 23 days old. When the rats were 25 days old their utricles were examined. This was made possible by using the biochemical marker of efferent neurons - calcitonin gene related peptide and coupling it to an immunoflourescent dye. At birth a few fibres were found below the epithelium at 2- 4 days. At 8 days there was a low density of fibres. It was found that there was no difference in the efferent innervation in the space exposed group when compared to Earth controls. Therefore the formation and major rearranging of efferent vestibular nerves occurs before day 8 in the rat. (Demmemes D et al 2001).

In the mouse the utricular hair cells undergo ultrastructural differentiation prior to synapse development. (Flock et al '73) (Van Der Water '76).

The cuticular plate has become well developed by day 19.

Two neurotropins, Brain derived neurotropic growth factor (BDNF) and neurotropin 3 (NT-3) and their high affinity receptors trk B and trk C are the sole support for the developing afferent inervation to the utricle. These neurotransmitters are expressed in the otocyst at the time when the afferent sensory neurones become post mitotic. (Fritzsch et al. 1999)

Otoconial development

The 1994 space shuttle with the onboard IML-2 (International microgravity laboratory -2) carried developing newt embryos in order to study the effects of gravity on the developing otoconia.

Growth and development of the otoconia appeared to be guided by environmenal gravity inferring the prescence of a feedback loop between the brain and the inner ear (Anken et al 2000).

The first otoliths are seen at approximately day 9 of development in newts.

These divide into utricular and saccular otoliths (Weiderhold et al 1995).

From the ILM – 2 experiments it appears that rearing in microgravity initiates a process which continues for several months in amphibians regardless of whether normal gravity is restored and the net result is the formation of larger and an increased volume of otoliths. One newt was kept for nine months upon its return to earth and held its head pointing upwards whereas the normal position of the head is horizontal. This suggests that even brief exposure to microgravity during vestibular dvelopment may have lasting consequences on an animal returning to the 1 G of earth (Weiderhold et al 1997).

It can thus be concluded that at least in amphibians there is a permanent effect of the basal rate of macular discharge due to gravity (the tonicity) on the position the head. Similarly exposure to hypergravity in a centrifuge leads to the development of significantly smaller otoliths (Anken et al 1999).

It has been suggested that the reason otlitiths form is due to the different relative concentrations of glycoproteins and proteoglycans in the gelatinous membranes of the

cupulae of the semicircular canals, the tectorial membrane of the organ of Corti and the maculae of the utricle and saccule. These differences may impart an ability to resist mineralisation or to mineralise. Interestingly the density of keratin sulphate almost doubles between days 7 and 21 days (Fermin et al. 1990). Other studies have shown that there is a gradient of increasing calcium density which is imparted to the otoliths by the gelatinous membrane in an increasing gradient from the vestibular surface to the otoconia (Campos et al. 1999).

Recent evidence suggests that there is an interaction of a protein - Otoconin 90 (the principal matrix protein) with microvescicles from supporting cells (Thalmann et al. 2001).

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Development of the hair bundle

This has been studied in some detail in humans (Dechesne and Sans ' 85) and in mice (Li ' 78), (Van Der Water ' 78), (Mbiene et al ' 84) and (Lim and Anniko ' 85). In humans it appears to start at day 14 and begins centrally, later extending to the periphery. Development in the crista lags behind the macula and progresses superoinferiorly. Initially the kinocillium is thicker and more off - centre than in the fully developed hair cell (Mbiene et al. '84) (Lim and Anniko '85).

Of the original 200 or so immature stereocillia in the immature hair bundle, only about 60 develop into mature stereocillia.

There has been the suggestion that there is transformation of normal microvilli covered cells to an immature hair cell bundle as described in the bullfrog (Lewis and Li '73).

By day 16 in the mouse there are two hair bundle types seen . Type 1 are taller (about 8 micrometers) and type 2 which are smaller (about 3 micrometers long).

This is in keeping with observations in amphibians. Here two hair cell bundle types are seen - Type LS (long stereocillia) and SS (short stereocillia) where the stereocillia are compared to the kinocilliary length. The sensory epithelium consists of a central zone containing all LS and some SS bundles and a peripheral zone containing only SS bundles.

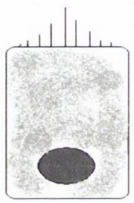
As development procedes the ratio of LS to SS bundles increases (Diaz et al. 1995)

Mechanism of hair bundle development

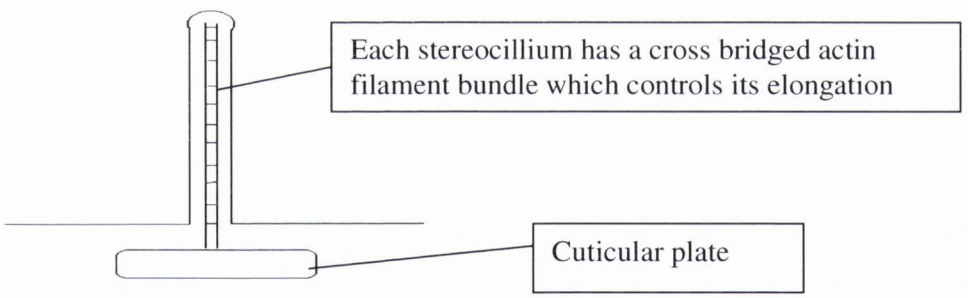
There are 4 phases of development as evidenced in the chick (Tilney et al ' 88).

Phase 1. Stereocillia sprout

Phase 2 An organpipe arrangement of the stereocillia occurs



Phase 3. Growth by elongation occurs.

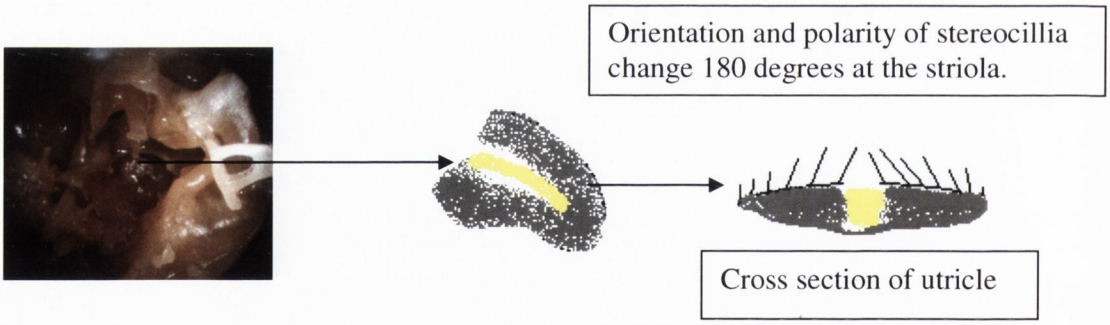


The initial elongation is followed by shortening and a kinocilliary bulb is thus formed. It has been suggested that the bulb formation is due to apical cytoskeletal depolymerisation and that this shortening may contribute to hair bundle reorientation as the inner ear develops. (Kelley MW et al 1995).

Phase 4. Maturity

In the chick inner ear the actin filaments are laterally situated in the stereocillia. As they become more centralised they form cross bridges and eventually form a hexagonal lattice.

Hair bundle polarity in the mouse is established in a definite manner. Utricles and saccules develop together in parallel. At embryonic day 13 the hair cell bundles appear. Initially they are not polarised and have a centrally placed kinocilluium at the cell surface. As the kinocillium becomes eccentrically placed polarisation is rapidly established. The polarisation is not random. By day 15 there is a definite demarcating line between oppositely polarised hair bundles in the maculae of both utricles. The line separates hair bundles which have polarities differing by approximately 180 degrees. Therefore this watershed line, the future striola is a line of orientation reversal.



At day 15 more immature hair bundles appear. Growth of both maculae occurs from the striola outwards and mainly when polarity and the watershed within the striola is established (Denman-Johnson K and Forge 1998) (Denman-Johnson and Forge 1999).

Hair cell development in the cochlea

Changes in the orientation of stereociliary bundles of hair cells in the cochlear sensory epithelium that occur during normal embryonic development and during the regeneration of hair cells that follows acoustic trauma have been observed. A common mechanism may guide reorientation both during embryonic development and during regeneration. Observations in living cochleae indicate that differentiating stereociliary bundles establish asymmetric linkages to the extracellular matrix of the developing tectorial membrane. During the growth of the tectorial membrane, its progressive extension across the surface of the sensory epithelium may exert traction forces through those asymmetric linkages that pull the bundles of the hair cells into uniform alignment (Cotanche and Corwin 1991).

Using transmission and scanning electron microscopy it has been observed that in both types of hair cells in the human foetus, synaptogenesis begins before ciliogenesis (week 10 of gestation versus week 12). In the inner hair cell (IHC), an adult like stage is seen around week 15 for synapses, but not before week 22 for stereocilia. In the outer hair cell (OHC), both synapses and stereocilia are not yet fully mature at week 22. Classic gradients of maturation are found: a base-to-apex gradient, and an inner hair cell to outer hair cell gradient. By comparing these results with the anatomical and functional data on cochlear development in laboratory mammals (Lavigne-Rebillard and Pujol 1990) the onset of cochlear function in the human fetus can be estimated to occur around week 18. The completion of cochlear maturation based upon the same anatomical criteria should occur about 10 weeks later.

Aminoglycoside ototoxcicity

Aminoglycoside antibiotics are now over 50 years old. They were discovered when streptomycin was isolated from cultures of Streptomyces Griseus in 1944 (Schatz et al. 1944).

This class of antibiotics were initially isolated from soil organisms. Other streptomyces species later yielded different aminoglycosides.

The development of streptomycin was the result of a planned search for antibiotics between 1939 - 1943, the bulk of the war years.

Once it was discovered and its cidal action was confirmed it was almost immediately noted that Mycobacterium Tuberculosis was sensitive to it (Waksman et al.1944) and it was quickly put to use as the first effective drug to treat this scourge of the ages.

It is now rarely used in clinical practice except for treating endocarditis, tularaemia and plague and as a second line drug therapy for Tuberculosis.

It does however still prove to be particularly useful in combating gram negative infections. Almost as soon as it was initially used the toxic effects on the inner ear were noted. Of an initial 34 patients treated for tuberculosis with the new antibiotic, one developed a transient deafness and three developed balance disorders (Hinshaw and Feldman 1945).

Another problem was early development of bacterial resistance and therefore attempts were made to administer the aminoglycosides with another agent when treating Tuberculosis. Resistance appears to be low today however with a rate of just 2-3% in isolates (Hardman and Lee 1996).

In a later treatment group 515 patients were treated for Tuberculosis.

8.2 % suffered adverse reactions one half of which involved the 8th nerve (both the auditory and vestibular functions), 2 % developed a rash and 1.4 % developed a fever. In 1949 histological evidence of toxicity to the organ of Corti was demonstrated (Berg and Causse 1949). Subsequent renal damage was noted shortly afterwards.

Gentamicin, today perhaps the best known of the family was the first aminoglyciside not isolated from streptomyces. It was isolated along with netilmicin from Micromonospora Purpura and Echinospora (Weinstein ML et al 1963). The different origin to these drugs is reflected in their different spelling from the other aminoglycosides (*micin* as oppossed to *mycin*).

In 1949 Waksman and Lechevalier isolated neomycin from Streptomyces Fradiae and in 1957 Kanamycin was isolated by Umegawa (Hardman and Lee 1996).

Tobrimycin and Amikacin were not isolated until the 1970s.

The continued problems with oto and nephrotoxicity led to a gradual decline in the use of aminoglycosides in most countries during the 1970s and 1980s (Begg and Barclay 1995) Despite this decline, of all the antibiotic classes aminoglycosides are today the most widely used worldwide (Begg and Barclay 1995) when one considers the various topical and parenteral preparations.

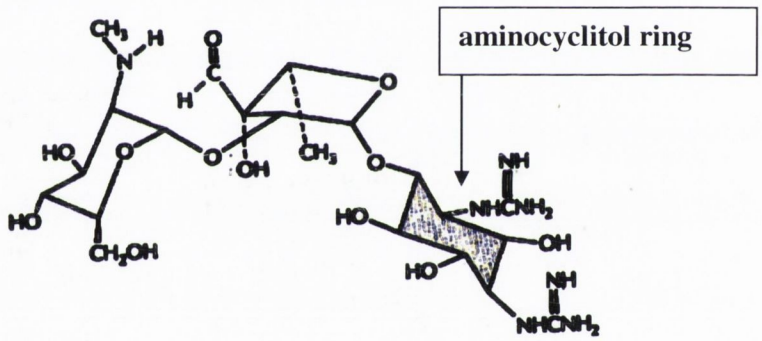
Their success and continued use can be attributed to their rapid bacteriocidal effect, synergism with beta lactam antibiotics, relatively low rate of resistance and their cheap cost.

Pharmacology

Aminoglycosides can be administered systemically, intramuscularly and topically.

The activity of aminoglycosides *in vivo* is cidal however intracellular organisms can escape attack. The cidal action of aminoglycosides is unusual as these drugs are inhibitors of bacterial protein synthesis and agents which attack by this means are usually static agents.

Aminoglycosides contain amino sugars linked to an aminocyclitol ring by glycosidic bonds.



They are polar and therefore oral absorption via the gut is universally poor.

Resistance is plasmid and transposon mediated (genetic) and there is also a decreased transport across the cell.

All aminoglycosides are ototoxic and nephrotoxic.

The drugs antibacterial action depends on decreasing translation of mRNA at Ribosomes (Shannon and Phillips 1982).

The resulting faulty proteins are inserted into the plasma membrane allowing more aminoglycoside into the bacterium (Bryan and Kwan 1995).

The precise mechanism for their rapidly lethal effect is currently unknown.

The drugs appear to diffuse through porin proteins in the outer membranes of gram negative and some gram positive bacteria to the periplasmic space.

Toxcicity

The use of aminoglycosides in cases of chronic supporitive otitis media places them into the very region of the body which is most sensitive to their toxic effects – adjacent to the inner ear, while systemic administration places both the inner ear and the kidneys at risk.

Since the 1950s we know that in the inner ear different aminoglycisides have different toxicity profiles with respect to the cochlea and vestibule.

Gentamicin for example, is less cochleotoxic than vestibulotoxic. The definition of cochlear toxicity is not uniform with respect to drugs however. Using a bilateral sensorineural hearing loss of 10 dB or more at one or more frequencies as has been advocated is not practical as often patients are too sick to be tested in sound proof booths. Using this somewhat unrealistic criterion there are reported ototoxicity incidences of 5 – 10 % with respect to gentamicin, amikacin and tobromycin.

Mechanism of gentamicin toxicity

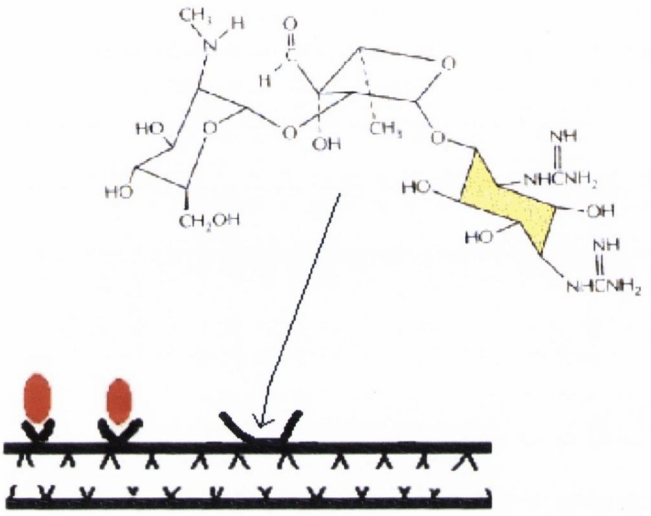
The reason why a particular aminoglyciside preferentially damages the cochlea or the vestibular apparatus is unknown.

The concentration of aminoglycoside in the inner ear fluids is persistently higher as the half life is 5-6 times as long in these fluids as in the plasma (Hardman and Lee 1996). The concentration of the drug in inner ear fluids is the same as it is in the fluids of other organs but it appears that they are much more slowly removed from the inner ear. Therefore the toxic effects seem not to be due to the accumulation of the drug in the inner ear fluids per se. (Tran Ba Huy P et al 1986).

The precise mechanism of aminoglyciside induced ototoxcity is unclear but the three stage model of Schacht ' 86 (Schact J 1986) seems to offer the best explanation.

Three stage model of Schacht

Stage 1.



The aminoglyciside molecule enters the environment of the cell and there is competitive binding of calcium and the aminogliciside at negatively charged receptors on the surface of the plasma membrane. This binding is fully reversible.

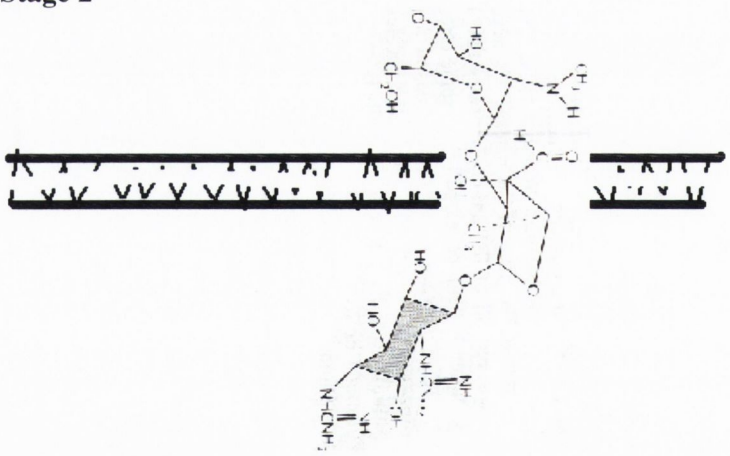
Some papers report up to 50 % recovery from cochlear toxicity which has been induced during treatment of an illness with aminoglycisides. This is hard to credit as no study to date has shown a recovery of damaged cochlear hair cells in an adult mammal. Perhaps these recoveries were due to a synergistic effect of the patients illnesses or more probably from the reversible effect of this first stage of aminoglyciside ototoxicity described above. The use of transient evoked otoacoustic emissions (TEOAE) to detect outer hair cell functional changes will almost certainly change our measuring of the true level of detection of the problem.

Otoacoustic emissions are measured by placing a probe with a seal in the ear canal.

Otoacoustic emissions are not electrical measurements but acoustic measurements which are measured in the ear canal with a tiny microphone. Clicks are transmitted into the ear and the first 3 milliseconds of the response is ignored as the stimuli traverse the middle ear apparatus. By analysing the response thereafter the response of the cochlea is identified. This data can even be broken down into frequency specific data. Transient evoked otoacoustic emissions can tell us about the quality of the cochlea, for example whether there is aminoglyciside induced damage.

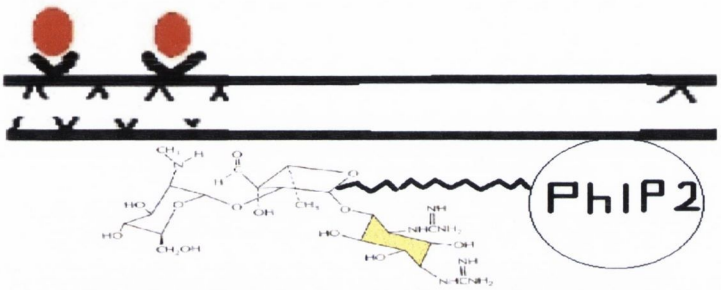
As far as measuring the changes in vestibular function, the problem is infinitely more difficult as there is an extremely wide range of variables for any standard test in the normal population.

Stage 2



Energy dependent transport into cell.

Stage 3 **2nd messenger stage**



This action interferes with a fundamental transmembrane signalling system which mobilises intracellular calcium and causes cytoskeleton contraction.

There is irreversible binding of the aminoglyciside to PhIP2 (Phosphatidylinositol bisphosphate). The binding occurs on the inner aspect of the plasma membrane. This prevents PhIP2 from being hydrolysed and therefore there is no biochemical response where there should be. Diacyl glycerol (DAG) and inositol phosphate (IP3) formation is prevented. Cell permeability increases and further aminoglycisides enter the cell. Cell death eventually occurs.

Other effects of aminoglycisides inside the cell include a prevention of polyamine induced cellular responses (Schact J 1986).

Aminoglycisides block hair cell calcium activated potassium channels and transduction channels, both reversibly and both with no obvious hair cell degeneration. Therefore this method of interference with the function of the inner ear must be independent of its long term toxic effects which are not reversible.

Aminoglycisides also bind to free iron to form free radicles. It has been shown that gentamicin ototoxicity in guinea pigs is decreased in the presence of free radical scavengers and iron chelators (Song and Scahact 1996)). Research into the

potential beneficial effect of taurine as a cytoprotectant is currently being undertaken.

Aminoglyciside effects on the peripheral vestibular system and the cochlea sensory cells are much more sensitive than supporting cells.

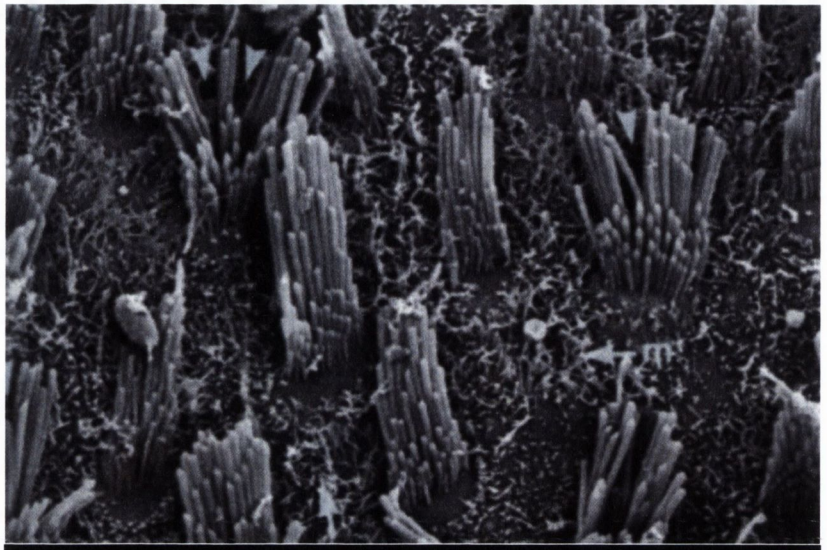
As previously mentioned different aminoglicisides are more potent than others at different ratios with respect to the vestibular aparatus and the cochlea. The pattern of effects is however the same.

Peripheral Vestibular Apparatus.

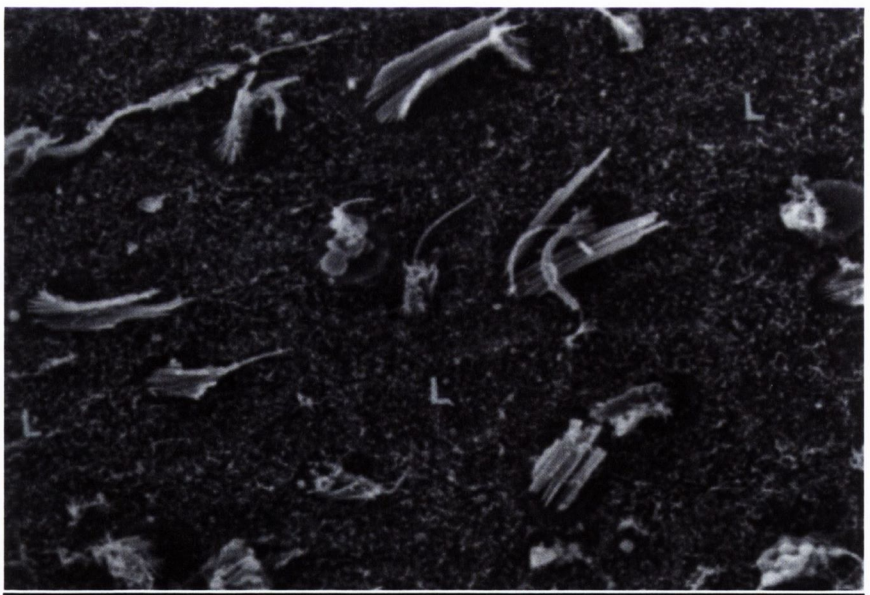
Hair cell loss is first seen in the striolar area of the macula of the utricle and saccule and the central part of the crista. It progresses radially from there with time.

Crista hair cell loss is greater than the utricle maclua hair cell loss which in turn is greater than the saccule macula hair cell loss.

Type 1 hair cells have a greater sensitivity than type 2 hair cells. Therefore regions which have a predominance of type 1 hair cells are more susceptible to damage than areas with a predominance of type 2 hair cells. The epithelium becomes flattened akin to an image of a first world war battlefield.



Scanning electron micrograph of the surface of the normal macula (Mag x 500)



Scanning electron micrograph of the surface of an aminoglycoside damaged macula (Mag x 500)

Both photographs reproduced with kind permission of Trinity College Dublin

Cochlea

The specific arrangement of the organ of Corti means that it can function as a highly sensitive organ to different frequencies and it also allows it to be highly frequency specific. These abilities are due to the existence of the cochlear amplifier.

It is possible that the cost of these abilities may be an inability to repair damage.

The outer hair cells bear the brunt of the damage inflicted by aminoglicisides. The row adjacent to the Tunnel of Corti is first to receive the insult. The damage progresses through the middle and outermost outer hair cells later.

The basal turn of the cochlea is most easily damaged and the damage spreads towards the apex from there depending on the dose and length of tretment. High frequency hearing loss therefore occurs first and with increasing doses and lengths of treatment spreads to the lower frequencies.

The inner hair cells last until their neighbouring outer hair cells die.

The order of cell death / damage is outer hair cells, Pillar cells, Deiter cells followed by Hensons cells. This is the same in vitro as it is in vivo and seems therefore to be due to an inherent tissue sensitivity and not an indication of the ease with which aminoglycosides can gain entry to the cells in question.

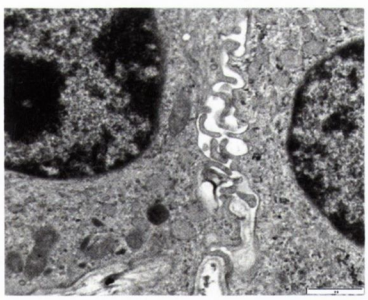
Interestingly even after chronic exposure to aminoglycosides for up to one month the tight junctions of the reticular lamina at the apex of the hair cells remains intact. In contrast the junctions between adjacent supporting cells become severely damaged (McDowell et al.1989).

Hair cell ultrastructural changes

These are the same in the cochlea and the utricle. The degree of damage is proportional to the particular aminoglyciside, method of administration and dose. First blisters appear on the apical surface of the hair cell and dense lipid bodies appear on the subcuticular region and along the sides of the hair cells.

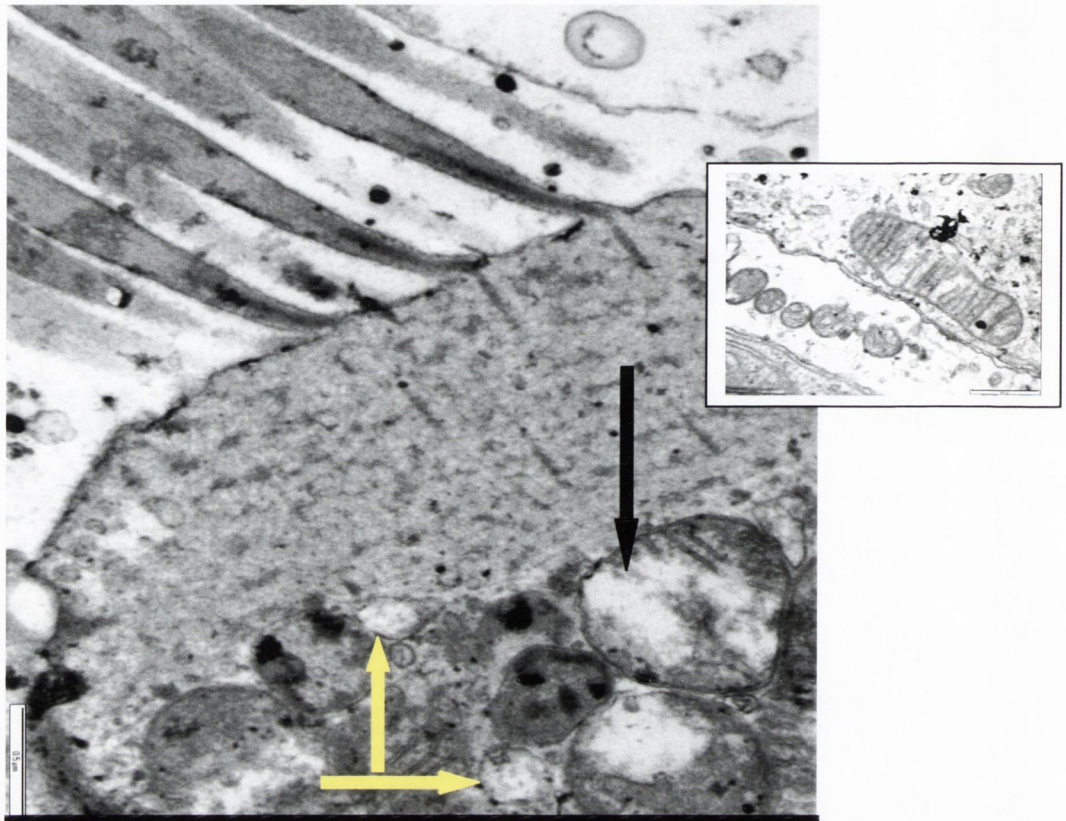
Early changes

The interspace between the pairs of membranes on the inside hair cells becomes irregular with fusing of regions to form a single dense layer.



Irregular interspace between cells

Mitochondria swell and accumulate lamellar bodies or disintegrate, ribosomes decrease in number, the endoplasmic reticulum accumulates and lysosomes and vacuoles form.



TEM showing mitochondrial swelling at the cuticular plate (black arrow) and vacuoles (yellow arrow) (X 5,000). A normal mitochondrium (inset) is shown for comparison.

Later the nuclear density increases, pyknosis occurs and cell disintegration occurs.

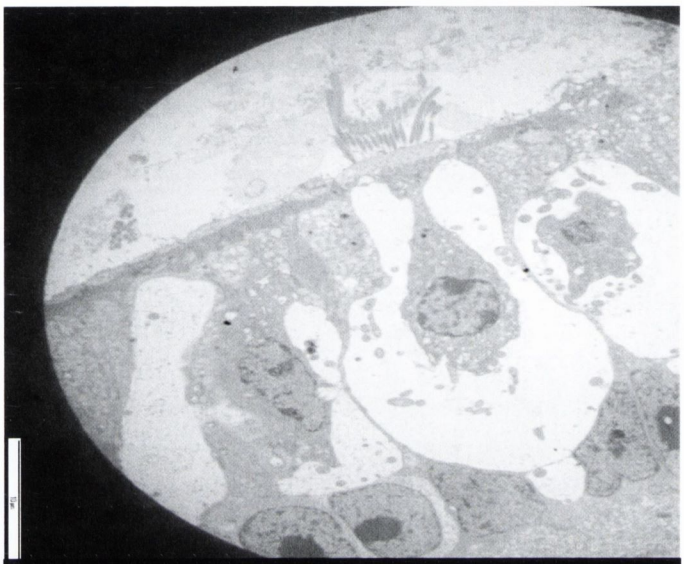
Stereocillia finally fuse, starting with the kinocillium.

There are two theories as to how this happens

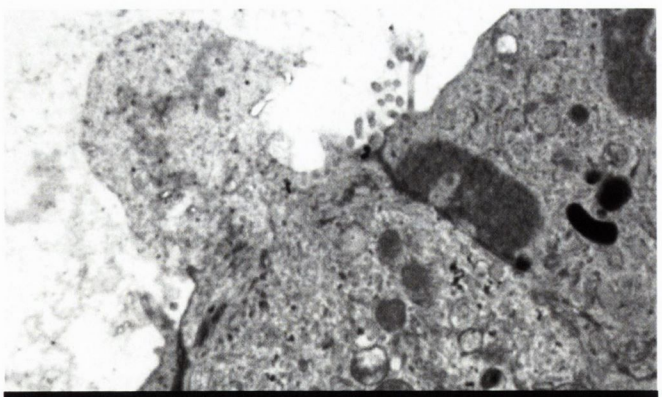
1. The cationic charge of an aminoglycoside antibiotic attaches to the negative charges on the surface of the hair stereocillia, so surfaces of adjacent stereocillia fuse.
2. Damage and subsequent loss of the hair cells glycocalyx.

Cell death

This occurs by apoptosis and by extrusion.



Transmission electron microscopy of a utricle showing apoptosis of an aminoglycoside damaged vestibular hair cell (Mag x 2,500)



Transmission electron microscopy of a utricle showing extrusion of an aminoglycoside damaged vestibular hair cell (Mag x 3,000)

Both processes are active and believed to be a specific repair attempt the inner ear has acquired to prevent further damage. The aim is to maintain the permeability barrier of the reticular lamina.

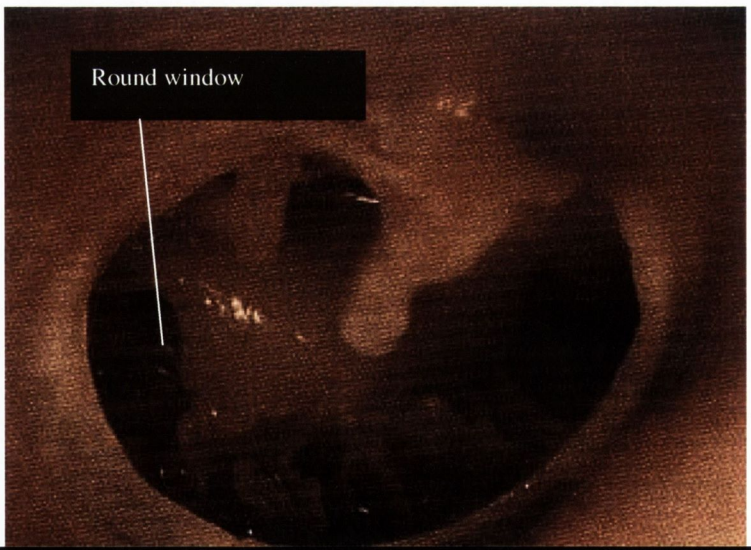
This separates the potassium rich endolymph at the cells apex from the relatively potassium poor perilymph which bathes the cell bodies and nerve fibres.

In the vestibule one study suggests that apoptosis predominates (Forge and Li L 2000). Where extrusion occurs, the apical fragment of the hair cell in the reticular lamina is retained until tight junctions are established between the expanded supporting cells.

Afferent nerves degenerate in a retrograde fashion. Efferent nerves stay for as long as several weeks.

Topical ear drops for chronic supporative otitis media

The use of aminoglyciside ear drops to treat chronic supporative otitis media has caused much debate. It seems ludicrous to instill toxic drops through a perforated eardrum into the very region of potential damage despite the fact that frequent offending microorganisms in otorrhoea of chronic otitis media include klebsiella, pseudomonas and proteus species which are sensitive to aminoglycosides. Transtympanic injection of aminoglycisides has caused complete destruction of hair cells in the inner ear of guinea pigs. The drug is absorbed through the round and oval windows to access the inner ear. The round window (and oval window) is thicker in humans and seems to prevent entry into the inner ear. Supporting this observation, the British Association of Otolaryngologists have succeeded in dampening the fear of using the drops for chronic supporative otitis media.



Perforation of the right eardrum of a patient where the round window can be seen. Any aminoglycoside drops which are instilled will be in direct contact with the membrane of the round window

Changes in dosing of aminoglycosides

Where the aminoglycoside is administered intravenously there has been a recent change in dosing regimes aimed at reducing their oto and nephrotoxicity.

Target concentrations with therapeutic concentration monitoring has been employed to minimise toxcity while maintaining adequate serum concentrations to maintain a cidal action. Recently dosing regimes have tended towards a larger single dose being given daily (Forge et al. 1996, Freeon CD et al 1997).

Numerous studies have established under different clinical settings that a once daily dose is as safe and as efficacious, costs less and is easier to administer.

Hair cell regeneration in fish and amphibians

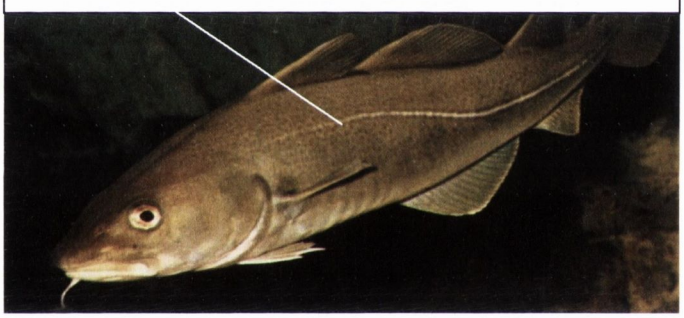
Introduction

Sensory hair cells are found in both the inner ear and in the lateral line of fish.

The lateral line runs down the side of a fish's body and relays information about both electrical currents and micro movements in the water about their bodies.

The line contains neuromasts which are discrete hair cell containing organs below the skin's surface.

Lateral line in the cod.



The lateral line is highly developed in the blind cave fish to compensate for total visual blindness in an environment where no light exists (Repass and Watson 2001). These cave fish depend almost entirely on the lateral line sensory system to detect nearby objects and when there are even minute vibrations in the water a response is initiated in which they swim more rapidly either towards prey or away from predators.

Cave fish



Because the lateral line and its neuromasts are so well developed in the cave fish it has served as a good model for examination of regenerating hair cells.

The neuromasts within the lateral line of fish are known to regenerate hair cells which have been lost and although the fish cannot hear through the lateral line it was reasonable to hypothesise that if the hair cells in the neuromasts can regenerate then so could the inner ear of fish regenerate.

The amphibians also possess the lateral line system and these animals can regenerate sensory cells here as well.

The mechanisms that lead to the production of sensory hair cells during regeneration have been investigated by using two different procedures to ablate hair cells in the sensory epithelia of individual neuromasts in the lateral line of of salamander tails, then monitoring the responses of the remaining cells (Balak et al.1990).



Lateral line in the tail of salamander.
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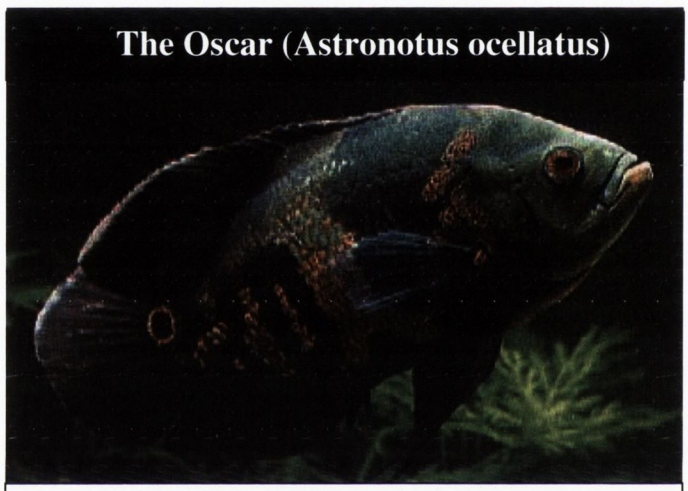
In one series of experiments, fluorescent excitation was used to kill hair cells. In another study the ultraviolet output of a pulsed laser was focused to a microbeam through a quartz lens and used to selectively kill individual hair cells. The cells were simultaneously imaged by transmitted light microscopy.

With the light microscope, observation of the treated neuromasts in vivo was possible. These experiments demonstrated that the mature sensory epithelia of neuromasts that have been completely depleted of hair cells can generate new hair cells.

Immediately after the ablations the only resident cells in the sensory epithelia were supporting cells. Therefore preexisting hair cells were not necessary for regeneration. The supporting cells were observed to divide at rates that were increased over control values, and eventually those cell divisions gave rise to progeny that differentiated into hair cells, replacing those that had been killed. Macrophages were active in these epithelia where removal of the destroyed hair cell debris was taking place, and their phagocytic activity had a significant influence on the population of cells. The first new hair cells appeared 3-5 days after the treatments, and additional hair cells usually appeared every 1-2 days for at least 2 weeks (Balak et al. 1990). The fate of the progeny produced by supporting cell divisions was plastic to a degree, in that these progeny differentiated either as supporting cells or as hair cells in epithelia where hair cells were missing or depleted.

It had thus been established that fish and amphibians can regenerate hair cells in the lateral line. This structure is a different organ to the inner ear, however it transpires that fish and amphibians possesses this regenerative ability with respect to hair cells in the inner ear also. This was demonstrated in the oscar fish (Astronotus ocellatus). Sensory hair cells in the striolar regions of this fish's utricle were damaged by intramuscular injections of gentamicin sulfate. In order to determine whether the fish could regenerate hair cells, the course of damage and recovery was followed over a period of four weeks by scanning electron microscopy of the removed utricles. Maximum loss of ciliary bundles occurred at about day 10 after the first of four daily injections of gentamicin. The striolar regions were almost totally denuded of ciliary

bundles, and there was considerable evidence of hair cell loss. The time course for damage was longer in larger fish, but the recovery of the ciliary bundles appeared to be complete about 10 days after maximal damage was seen. This indicated that the oscar is able to repair damage to inner ear hair cells in the post-embryonic period (Lombarte et al.1993).



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Advancing one step up the evolutionary tree from fish to the amphibians, regeneration of hair cells in the inner ear is also observed. In the bullfrog which has been treated by ablation of the inner ear with aminoglicosides, the vestibular system has regenerated new hair cells also. Interestingly the regenerated hair cells in the bullfrog developed an afferent nerve supply . These nerves have since been shown to be active when stimulated and this supports the notion of functional and not just structural recovery of the sensory organ (Hernandes et al. 1995).



Bullfrog

It transpires that the hair cells in the inner ear and lateral line in fish and amphibians are continuously produced throughout life (Corwin 1981). These regenerated cells arise from supporting cell mitosis. For instance eighty percent of the cells in the macula neglecta of the shark are produced postembryonically (Corwin 1981). This allows improved sensitivity of hearing as the shark matures. This means that fish and amphibians have a distinct advantage over mammals in terms of potential to self repair damaged or destroyed hair cells.

Phylogenetically fish evolved into amphibians, amphibians into reptiles and subsequently reptiles into birds. The ability to repair damaged hair cells in the post embryonic period has been retained throughout this lineage.

Hair cell degeneration and subsequent regeneration in birds

The organ of hearing in avian species is the basilar papilla, the equivalent of our organ of Corti. It consists of a regular mosaic like make up of hair cells and supporting cells. It contains both long and short hair cells. Like our organ of Corti it is damaged by excessive sound exposure.

Excessive noise causes predominantly short hair cell loss in an area of the papilla related to the frequency of the sound (tonotropic distribution). Damage, as in humans is related directly to the duration and intensity of the sound (Cotanche et al. 1994). The degenerative sequence following acoustic trauma is as follows. The damaged hair cells are either extruded or undergo apoptosis. The tectorial membrane is damaged which exposes the underlying sensory epithelium at the site of damage. Furthermore the endocochlear potential is reduced. There is hair cell loss across the basal half of the basilar papilla with a corresponding high frequency hearing loss (Cotanche 1994).

Aminoglyciside induced damage is mediated in a different way. It does not affect the tectorial membrane, damages both long and short hair cells and is much slower to recover, taking 2 – 20 weeks depending on the aminoglycoside (Hashino et al.1991). For example gentamicin induced damage takes approximately 10 – 20 weeks to recover (Duckert and Rubel 1993) while kanamicin induced damage takes about 2 weeks to fully recover (structurally) (Hashino et al. 1991).The reason for the difference in response to the different aminoglycosides is that hair cell recovery starts basally and progresses apically. There is a delay in the formation of a kanamicin transmembrane transport system so even though the kanamicin remains locally for some time, it cannot be transported into the cell (Hashino and Salvi 1996).

Hair cells can recover a second and third time after noise induced or aminoglyciside induced damage. The degree of loss after a second kanamicin induced damage is less than that initially experienced, however results of studies suggest metabolic changes occur in response to the initial dose of kanamycin which do not necessarily involve changes in hair cell resistance to ototoxicity so the resistance may be systemic.

Birds, as stated, regenerate auditory hair cells when original hair cells are lost.

The regenerative sequence is as follows.

Regeneration of the tectorial membrane usually begins within 24 hours. At 10 days there is a honeycomb like matrix present which fuses with both the adjacent normal tectorial membrane and establishes a connection with the stereocillia of the newly forming hair cells (Cotanche 1987 (b)).

Regeneration of hair cells in birds was first discovered in 1987 (Cotanche 1987 (a)) Immature looking hair cell were discovered in the inner ear following noised induced hair cell loss. Subsequently the same observations were made after aminoglyciside induced hair cell loss (Cruz et al. 1987).

This was an unexpected finding as the inner ear of birds unlike fish and amphibians was believed to be quiescent after embryonic development (Katayama and Corwin 1989). The subsequent discovery that there was functional recovery was greeted with excitement.

As in fish and amphibians studies, avian studies have shown that the reason there is functional recovery is the formation of regenerated neural connections to the regenerated hair cells. One study found regenerated hair cells identified by autoradiography ([3H]thymidine) in ears from mature quail exposed to pure tone overstimulation. When analyzed for their neural contacts using transmission electron microscopy the results provided conclusive evidence of both efferent and afferent

synaptic regeneration on regenerated hair cells of all types 10 days following the trauma (Ryals and Westbrook 1994).

In a separate study forty day old quails were given kanamycin daily for ten days. Evoked potential thresholds were obtained at 1 day or 1, 2, 3, 4 and 6 weeks respectively after the last injection, and compared with the thresholds of age-matched control animals. The cochleas of the animals were removed and examined by scanning electron microscopy (SEM). The results showed that the application of kanamycin for 10 days produced massive destruction of the basilar papilla at 1 day post treatment. The damaged region began at the base and extended apically to the position about 50-70% of the total distance along the papilla, with most severe damage in the basal end. The auditory thresholds measured at the same time revealed a significant elevation at 2 and 4 kHz. However, the number of hair cells in the damaged region recovered rapidly with time, and so did the function. By the end of 3 weeks post injection, almost all the hair cells reappeared in the damaged region and the function improved further, with nearly normal thresholds at all frequencies but 4kHz. Six weeks after treatment, the basilar papilla showed a nearly normal appearance. The results of the study demonstrated that structural recovery was faster than functional one (Lou et al. 1994).

At least one study however suggests that a direct comparison of structural and functional recovery indicates that auditory thresholds recover maximally before a full complement of hair cells has regenerated in Bengalese finches (Woolley et al. 2001).



Bengalese finch

It has been established that the regeneration of inner ear hair cells and neural connections to these hair cells following trauma is therefore accompanied by a repair of the sensory organ as a functional unit (Lou et al. 1994).

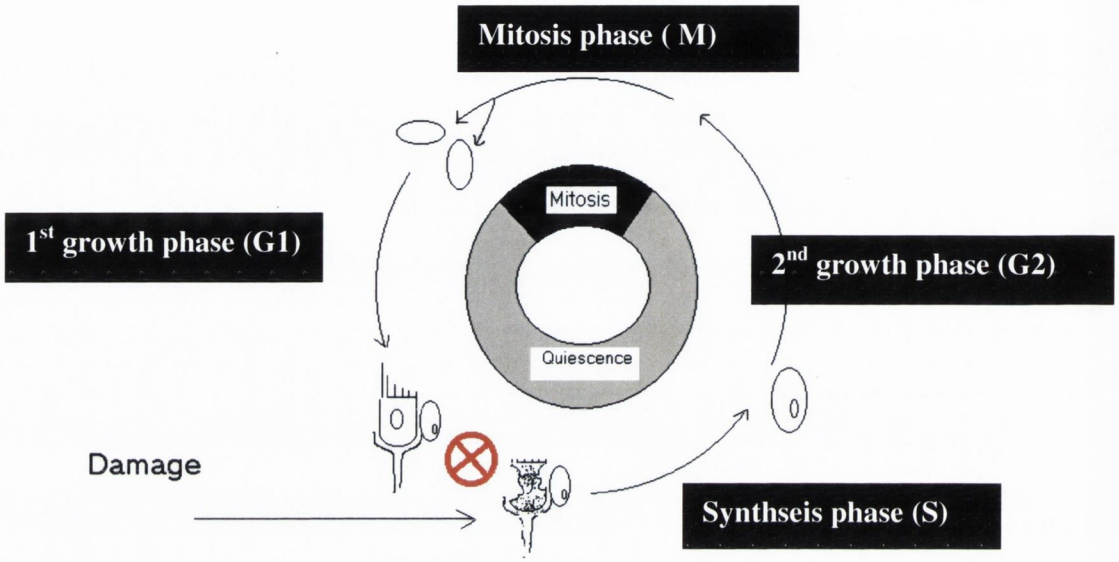
Put simply the results of regeneration correspond to a functional recovery.

Functional recovery during hair cell regeneration is particularly interesting in animals that depend on hearing for communication and perhaps this dependence on hearing for identification of a mate and other forms of communication in avian species has meant that regeneration of inner ear function following trauma was a distinct advantage for survival and propogation of the species.

Once it was established that inner ear hair cell regeneration in mature birds occurred the next logical question was where did the regenerated hair cells originate ?

It is now known that postembryonic production of sensory hair cells occurs both in normal and aminoglycoside-damaged avian inner ears (Jorgensen and Mathiesen 1988), (Robbertson et al. 1992). If hair cells are formed at a steady rate under normal circumstances, the origin of the new hair cells was almost certainly going to be the same cells from which regenerated hair cells originate.

The cellular source and mechanism that resulted in new hair cells was investigated in the avian vestibular epithelia using three distinct cell-cycle-specific labeling methods to identify proliferating sensory epithelial cells.



The cell cycle. Supporting cells can reenter the mitosis / differentiation cycle

First, immunocytochemical detection of the proliferating cell nuclear antigen, an auxiliary protein of DNA polymerase, allowed labeling of cells in late G1, S, and early G2 phases of the cell cycle.

Second, a pulse-fix tritiated thymidine autoradiographic protocol was used to identify cells in S phase of the cell cycle.

Finally, Hoechst 33342, a fluorescent DNA stain, was used to identify epithelial cells in mitosis. The distribution of cells active in the cell cycle within the normal and damaged vestibular epithelium suggested that supporting cells within the sensory epithelia are the cellular precursors to the regenerated hair cells. Differences between the proliferation marker densities in control and damaged end organs indicated that the upregulation of mitotic activity observed after streptomycin treatment was due primarily to an increase in the number of dividing progenitor cells.

The differences between the extent of ototoxic damage and the level of the reparative proliferative response suggested a generalized stimulus, such as a soluble chemical factor, plays a role in initiating regeneration. Finally, after DNA replication was initiated, progenitor cell nuclei migrated from their original location close to the basement membrane to the lumenal surface, where cell division occurred.

This pattern of intermitotic nuclear migration was analogous to that observed in the developing inner ear and neural epithelium (Tsue et al. 1994).

A further experiment measured the changes that occurred in expression of mRNA for the hair cell-specific cytoskeletal proteins fimbrin and class III beta-tubulin, along with that for beta-actin, in the utricle of chicks after gentamicin and neomycin induced hair cell damage both in vitro and in vivo.

The total RNA was extracted from single utricles, reverse transcribed to cDNA and the cDNA amplified by PCR for beta-actin, fimbrin and class III beta-tubulin. Co-amplification allowed quantitative comparison of fimbrin, class III beta-tubulin and beta-actin mRNA from the same utricle.

The experiment concluded that the cells of the sensory epithelium of the chick utricle subjected to aminoglycoside-induced damage undergo a process in which mRNA expression is switched away from the production of functional proteins and towards proteins necessary for structural re-organisation. Therefore there must be up regulation of hair cell production relative to the basal rate of hair cell turnover following the damage (Stacey and McLean 2000).

What causes this up regulation in birds ?

Messenger RNAs (mRNA) of several growth factor receptors and relate genes were examined with reverse transcriptase polymerase chain reaction in normal and noise-damaged chicken basilar papillae. Analysis of the amplification products indicated the presence of mRNAs for epidermal growth factor receptor (EGFR), fibroblast factor receptor (FGFR), insulin-like growth factor receptor (IGFR), insulin receptor (IR), retinoic acid receptor beta (RAR beta), retinoic acid receptor gamma (RXR gamma), and basic fibroblast growth factor (BFGF) in both normal and noise-damaged BP. The PCR products generated were characterized by size and sequencing analysis to confirm the identities of the target molecules. The subcellular localization of the mature protein analogs for EGFR, FGFR, IGFR,RAR beta, and BFGF were identified using fluorescence immunocytochemistry and confocal laser scanning microscopy.

These experiments indicated that EGFR is present in the stereociliary bundles in the hair cells, IGFR is not present in the cells of the basilar papilla, BFGF localizes in the nuclei of supporting cells in the basilar papilla, but not hair cells, and that RAR beta localizes in the perinuclear regions of hair cells. The subcellular distributions of these proteins were consistent in both noise-damaged and control basilar papilla. FGFR, in contrast, changed its distribution in the tissue after noise damage. In normal basilar papilla, FGFR is concentrated in the stereocilia of hair cells. However, in damaged regions of noise-exposed chick cochleae, FGFR was heavily expressed in the expanded apical regions of the supporting cells. These findings suggest that BFGF and retinoic acid may potentially play a role in the mechanisms which regulate the regeneration of chicken cochlear hair cells (Lee and Cotanche 1996).

Functional recovery in birds

Following noise induced hair cell damage, auditory thresholds and frequency selectivity returns within 3 days after exposure in line with the first appearance of the immature hair cells (Nimiec et al.1994). As the tall hair cells appear to be unaffected, the recovery of the tectorial membrane overlying them allows these hair cells to be stimulated when the basilar membrane is moved (Saunders et al. 1992).

Almost all the innervation of the basilar papilla is from the tall hair cells.

Aminoglyciside damage damages both the tall and the small hair cells. Therefore functional recovery takes much longer, depending on the particular aminoglycoside, again gentamicin taking far longer than kanamicin. Functional recovery depends on hair cell recovery. There is however a continuous deficit in function such as a high frequency defcit in chicks due to disorientation in the arrangement of the hair cells at the basal end of the papilla (Marean et al.1995)

Curiously this is a low frequency deficit in the budgerigar (Hashino et al.1991).

Vestibular system in birds

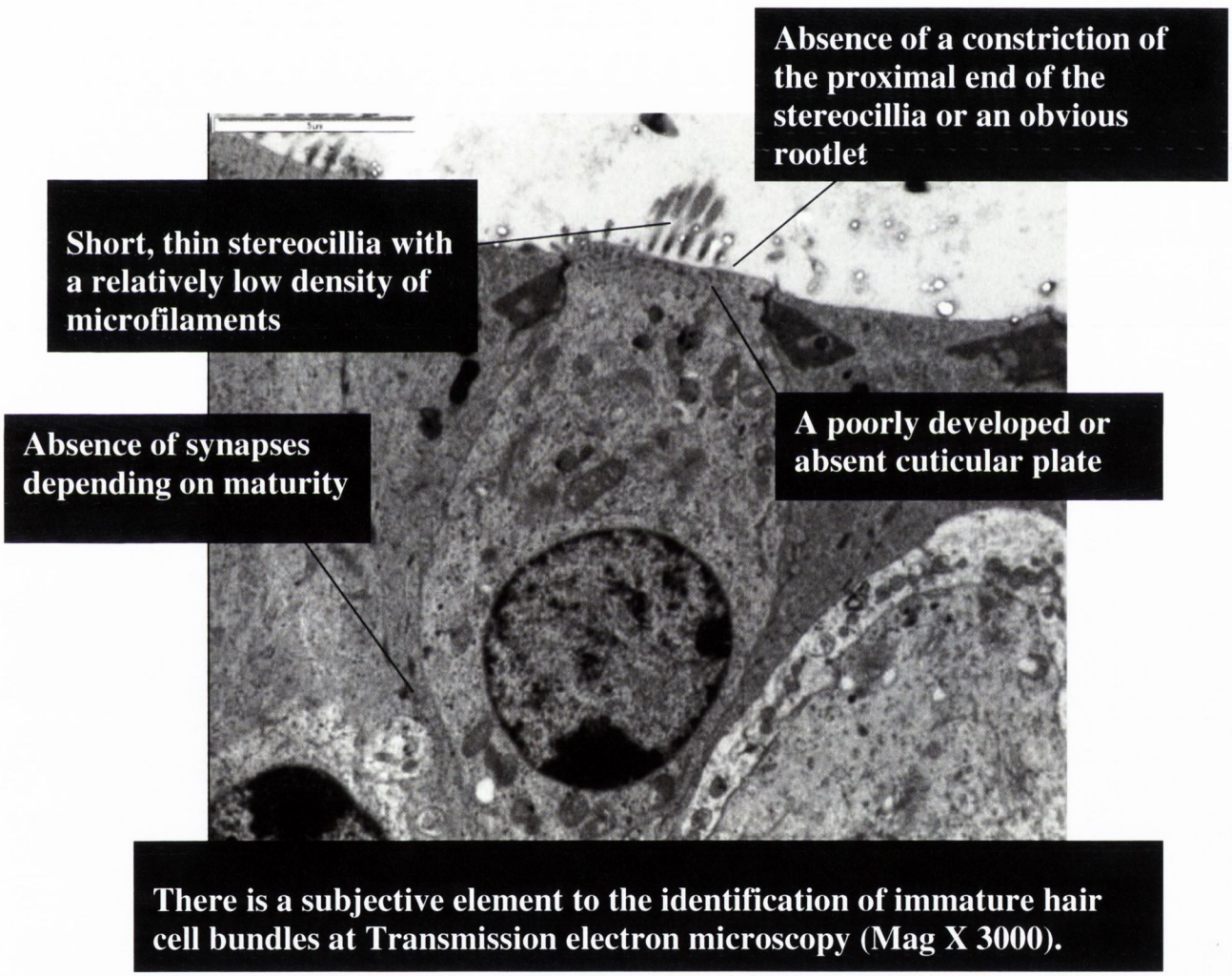
The avian vestibular system is structurally, developmentally and functionally similar to the mammalian vestibular system. The vestibular sensory epithelium does not continue to grow after the embryonic period but hair cells are produced (unlike the auditory system) in the postembryonic period (Jorgensen and Mathiesen 1988.) and (Robbertson et al. 1992). Therefore there must be a turnover of hair cells in the vestibular system. Ongoing supporting cell proliferation in the post embryonic period has been shown (Osterle and Rubel 1993).

There is a functional recovery of the avian vestibular system after aminoglyciside induced damage and vestibular evoked potentials are normal at 10 weeks whereas the vestibulo – ocular reflex returns at 8 – 9 weeks (Jones and Nelson 1992).

Mammals

The Immature hair cell bundle

The regenerative hair cell unit of the mammalian utricle is the immature hair cell bundle.



This ensemble gets its name from the fact that it resembles the immature hair cell bundles seen in normal development of other mammals.

The auditory epithelium in mammals has evolved separately from that in birds and its structural organization differs from those of lower vertebrates. In contrast, the vestibular epithelia, present in the saccule, utricle, and the cristae of the three semicircular canals, are morphologically similar in all vertebrate classes. These similarities have led to the speculation and discovery that hair cell regeneration takes place in the mammalian vestibular system (Forge et al.1993).

The most widely studied sensory epithelium is the utricle. Large number of cells with immature hair bundles in multiple stages of development can be identified in the utricle of guinea pigs which has experienced aminoglycoside induced hair cell loss. Thin sections show that lost type 1 hair cells were replaced by cells with a morphology similar to that of type 2 hair cells. These results indicated an unexpected capacity for hair cell regeneration in vivo in the mature mammalian inner ear. At least some of the new hair cells become innervated, making it likely that they could contribute to a recovery of sensory function (Forge et al.1993).

Similar observations have been made in cristae and saccules,

which suggests that these phenomena occur throughout the whole mammalian vestibular system.

The structure of an immature hair cell bundle consists of short thin stereocillia of almost equal height and a single longer koinocillium.

The most immature looking consist of these stereocillia which cover almost the entire cell apex and are angled towards a central kinocillium. The apical surface is smaller than that of mature hair cells. The more mature immature hair cell bundles have regularly arranged stereocillia which are longer and have cross links at their tips. They

have an eccentrically placed kinocillium.

The immature hair cell bundles are found in greatest numbers 4 weeks after treatment with gentamicin (Forge et al.1993).

Their densities decline in the following ten months (Quint 1996).

There is a subjective element to the identification of immature hair cell bundles.

TEM of the immature hair cell bundles show

1. Short, thin stereocillia with a relatively low density of microfilaments
2. Absence of a constriction of the proximal end of the stereocillia or an obvious rootlet
3. A poorly developed or absent cuticular plate
4. Absence of synapses depending on maturity

Innervation of the immature hair cell bundles has been confirmed. Hair cell counts of thin sections suggest that there is a recovery of hair cell mumbers over 12 weeks. The number of type 1 hair cells increases with time. (Forge and Li 1994)

The origin of the immature hair cell bundles in mammals is unknown and currently the mechanism underlying the regenerative process is still under debate.

There are four possible sources.

1. **Supporting cell mitosis (Warchol)**

   The main evidence to support this theory comes from cultures of mature guinea pig and human utricles which had been exposed to neomycin in titrated thymidine and subsequently showed labled nuclei in the basal part of the sensory epithelium at 2 – 6 days following treatment. These cells were consistent with supporting cells. However by 4 weeks a proportion had migrated to the lumenal stratum where the hair cells would be found. Some of these labled cells subsequently developed immature hair bundles (Wachol et al. 1993).

2. **Transdifferentiation of supporting cells or stem cells**

   This theory is based on the belief that immature hair cell bundles arise from transformation of another cell type. The evidence in support of this theory is based on the observation that following transtympanic injection of gentamicin into guinea pig ears the number of immature hair cell bundles was considerably greater than the number of labled supporting cells seen on Scanning Electron Microscopy and the nuclei of the immature hair cell bundle cells were not labled with tritiated thymidine.

3. **A combination of 1 and 2 (Sobkowicz)** (Warchol et al.1993),(Rubel et al.1995).

4. **Cell repair (Sobkowicz) (Zheng).**

Intracellular repair of partially damaged hair cells can be an important contributor to spontaneous hair cell recovery in mammalian inner ears. Immunocytochemical, histological, electron microscopic, and autoradiographic evidence in cultured postnatal rat utricles suggest that a substantial number of hair cells can survive gentamicin insult and regrow their stereocilia (Zheng et al.1999)

Although the number of stereocilia-bearing hair cells increases over time after gentamicin insult, hair cell and supporting cell numbers remain essentially unchanged. Tritiated thymidine autoradiography and bromodeoxyuridine immunocytochemistry of the cultures demonstrated that cell proliferation in the sensory epithelium was very limited and far below the number of recovered hair cells. This seems to support the previous studies (Rubel E.W, Dew, L.A. et al 1995).

Further evidence for self recovery of hair cells by repair comes from studying the kinocillia in cell cultures.

Auditory hair cells of mice that survived mechanical injury in culture began their recovery by reforming the kinocilium. Normally, the kinocilium lasts only about 10 postnatal days; however, post-traumatic hair cells reformed their kinocilia regardless of age. Concomitant with the regrowth of the kinocilium, the basal body and its cilium took a central location in the cuticular plate and the stereocilia regrew. The reformation of the kinocilium in recovering hair cells indicates the possible role of its basal body in the morphogenesis and differentiation of cuticular plates and stereocilia (Sobkowicz et al.1995)

Considered together, intracellular repair of partially damaged hair cells can be an important contributor to spontaneous hair cell recovery in mammalian inner ears.

Functional recovery in mammals

Evidence for functional recovery comes from two sources.

1. Nystagamus and decarboxylase activity have been shown to return in guinea pigs treated with streptomycin at 22 days (Meza et al 1992.)

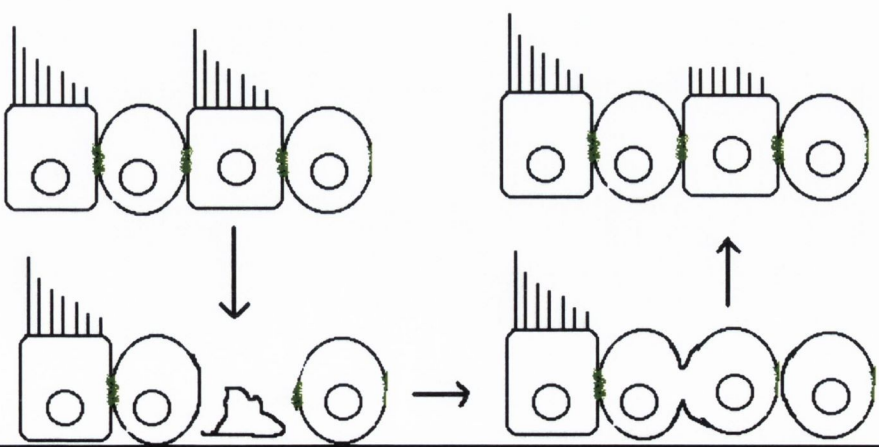
2. The return of vestibular function in an ear treated with streptomycin for Menieres disease in a patient (Glascock et al. 1989).

What is the up regulating stimulus for hair cell regeneration ?

There are currently two theories as to the origin of the regeneration stimulus.

Removal of lateral inhibition (Corwin, Lewis).

Examination of the vestibular sensory epithelium reveals a classical alternating hair cell / supporting cell arrangement. The junctions between neighbouring cells possess signalling molecules which are believed to mutually inhibit the growth of a cell identical in function to itself. When hair cells are lost this means that a supporting cell must divide and differentiate into a new hair cell to restore the balance (Corwin et al. 1991), (Lewis 1991). Avian vestibular hair cell regeneration has been demonstrated in serum free conditions and this supports this theory (Osterle et al. 1993)



Removal of cellular lateral inhibition may allow supporting cells to re-enter the division stage of the cell cycle

Growth factors in the epithelium (Contache)

This theory suggests that a sensory epithelium produced growth factor induces mitosis and differentiation of supporting cells into hair cells. Avian vestibular hair cell regeneration has been demonstrated in serum free conditions and this is why the origin of the growth factor is believed to be in the epithelium itself (Cotanche 1997) The proliferative process is mediated by cAMP (Navaratam et al. 1996).

Growth factors specific to mammalian vestibular hair cell regeneration following aminoglyciside induced damage include transforming growth factor alpha (TGFA) and Epidermal growth factor (EGF) which both mediate their effects through the EGF receptor (Yamashita and Oesterle 1995).

TGFA is much more potent than EGF. The combination of TGFA, insulin growth factor 1 (IGF –1) and retinoic acid increased the rate of hair cell regeneration in vivo in the guinea pig utricle (Lefebvre et al. 1993).

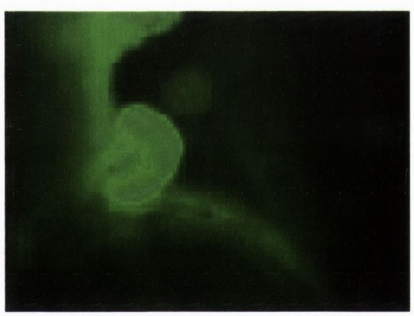
Recently the list of mammalian vestibular growth factors has expanded.

Brain derived neurotropic factor (BDNF) (Lee et al 1997), Fibroblast growth factor 2 (FGF – 2) (Zheng et al. 1997) and Recombinant human glial growth factor 2 (rhGGF –2) (Gu et al. 1997) are all implicated in the growth of immature hair cell bundles.

Stem Cells and Mammalian Inner Ear Regeneration

The recent interest in stem cell research has yielded some promising results in the field of inner ear regeneration in mammals. It was known that foetal dorsal root ganglion neurons can extend functional connections in the rat spinal cord. Embryonic stem cells and adult neural stem cells have also shown the potential to differentiate into neurons.

Embryonic foetal mouse stem cells from the dorsal root ganglia and adult mouse neural stem cells have been injected into the injured vestibulocochlear nerve of adult rats and guinea pigs. The foetal cells were labled with Enhanced Green Fluorescent Protein (EGFP) and the adult neural stem cells with lac Z reporter genes. Survival of the implants at 2 to 4 weeks later has been assessed.



EGFP identifying a stem cell. Reproduced with permission of University College Dublin

By labelling the embryonic stem cells with the mouse-specific neuronal antibody Thy 1.2. and by looking for lacZ-expressing adult neural stem cells the stem cells were identified as having differentiated into neuronal tissue.

EGFP-positive nerve fibers were seen growing within the proximal vestibulocochlear nerve at the site of injection but also in the brain stem in both the rats and guinea pigs. (Regala C, Duan M, et al. 2005).

This suggests that the vestibulocochlear nerve of adult rats and guinea pigs will support xenotransplants of embryonic dorsal root ganglia and embryonic stem cells. The same should apply to adult neural stem cells.

In addition to their innate potential to reform damaged neuronal tissue, the ability of nerve stem cell-derived cells to produce neurotrophins in the inner ear has also been demonstrated (Nakagawa T, Ito J. 2005). Results from studies using autologous bone marrow stromal cells indicate a high survival and migration potential also suggesting that these cells can be used as a drug delivery vehicle to the inner ear. These cell transplantation findings may provide a sound foundation for the development of therapies to treat inner ear disorders.

To more efficiently drive the implanted adult mouse neural stem cells into a neuronal fate when transplanted into damaged guinea pig inner ears nerve stem cells have been transduced with neurogenin 2 (ngn2) before transplantation. The surviving cells were found close to the sensory epithelium and adjacent to the spiral ganglion neurones and their peripheral processes (Hu Z, Wei D et al 2005).

It is not just nerve growth factors which show promise in manipulating implanted stem cell. By profiling gene expression in developing mouse vestibular organs, the retinoblastoma protein (pRb) was identified as a possible candidate regulator of cell cycle in hair cells (Sage C. Huang M 2005).

Sage et al have shown that differentiated adult mouse hair cells with a targeted deletion of Retinoblastoma 1 gene (Rb1) undergo mitosis, divide, and cycle to become highly differentiated and functional. Acute loss of Rb1 in postnatal hair cells caused cell cycle re-entry. Manipulation of the Rb pathway may ultimately lead to mammalian hair cell regeneration.

Taurine as an immunomodulator and cellular protectant

Taurine or β - aminoethane sulfonic acid is a β -amino acid with plasma concentrations of 74 + / - 27μL in humans (Vinton et al '86). This is similar to other plasma amino acid concentrations. The intracellular concentration however is very high, up to ten times higher than the other amino acids.

Total body taurine is as much as 100 – 150 mmol. Fifty percent is present as a free amino acid pool in the heart. Large amounts are also found in the skeletal muscle, brain and bile.

It is found in high concentrations particularly in mammalian electrogenic tissue (Huxtable, Sebring 1986). From here it is taken into platelets and leukocytes and transported to organs.

Taurine is an essential amino acid which is not utilized in protein synthesis, but rather is found free or in simple peptides. Clinically, taurine has been used with varying degrees of success in the treatment of a wide variety of conditions, including: cardiovascular diseases, hypercholesterolemia, epilepsy and other seizure disorders, macular degeneration, Alzheimer's disease, hepatic disorders, alcoholism, and cystic fibrosis (Birdsall TC. 1998)

In humans taurine is absorbed through the gut and plasma levels are controlled by the kidney. An efficient uptake system is required to overcome the low dietary intake of taurine. In the gut, the intramucosal concentration of taurine is 90 times the plasma concentration. The overwhelming majority however is produced by the body itself. As it is made chiefly in the liver and brain and remains for the most part intracellular, the membrane transport system in the gut must be responsible for maintaining the high intracellular mucosal concentrations there.

The small portion of taurine obtained from the diet comes almost exclusively from animal foodstuffs - meat, offal and seafood.

Taurine was initially viewed as an end product of sulphur containing amino acid metabolism with no physiological role except bile conjugation (Hastlewood '64).

Now its complex physiological role is being more completely understood.

It has a role in many different cellular protective mechanisms such as

1. Osmoregulation (Heacock AM, Kerley D. et al. 2004) (Ito T, Fujio Y et al. 2004)
2. Antoxidation (Bidri M, Choay P. 2003)
3. Detoxication (Birdsall TC. 1998)
4. Membrane stabilisation (Gaull and Rossin '79) (Huxtable '92)
5. Neuromodulation (Kuriyama et al '80) (Barbeau A (1975)).
6. Retinal and cardiac function (Wright et al ' 86)
7. Brain development (Sturmann et al '86)
8. Immunity (Watson et al 1995)

Immunity

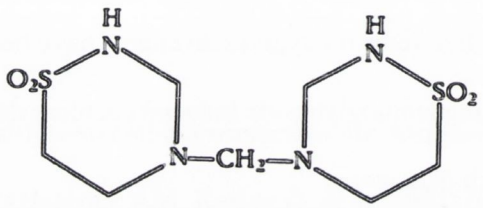
Given taurine's established role in membrane stabilisation, osmoregulation and antoxidation, it is reasonable to suggest that it has a positive effect on cell viability. The effects of taurine on apoptosis and necrosis has been studied.

In hepatocytes taurine has attenuated apoptosis and reactive oxygen intermediate induced necrosis. Since free redical oxygen scavengers have been shown to have some effect on modulating aminoglycoside induced cochlear damage, then in theory taurine may prevent such damage.

It has also attenuated apoptosis related to nitric oxide inhibition and attenuation of necrosis caused by free radical concentrations has been described.

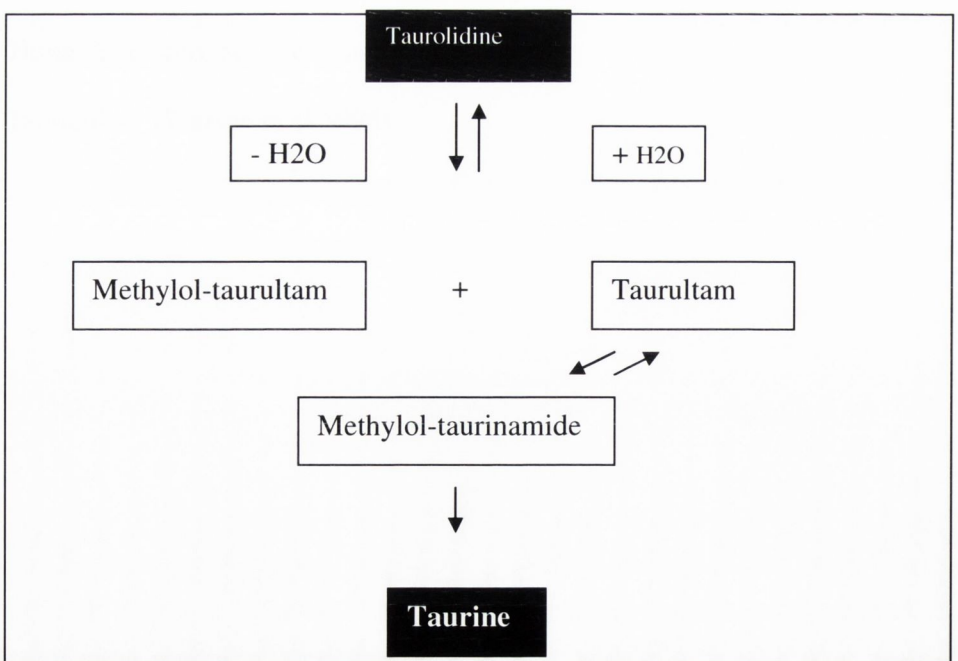
Basic science

Taurolidine is Bis (1,1, Dioxoperhydro – 1,2,4 – thioiazinyl – 4) methane. The chemical structure is $CH_2H_{16}N_4O_4S_2$ and the molecule has a molecular weight of 284.36. It has a pH of 6.9 – 7. and is a white crystalline powder. It is up to 2% water soluble.

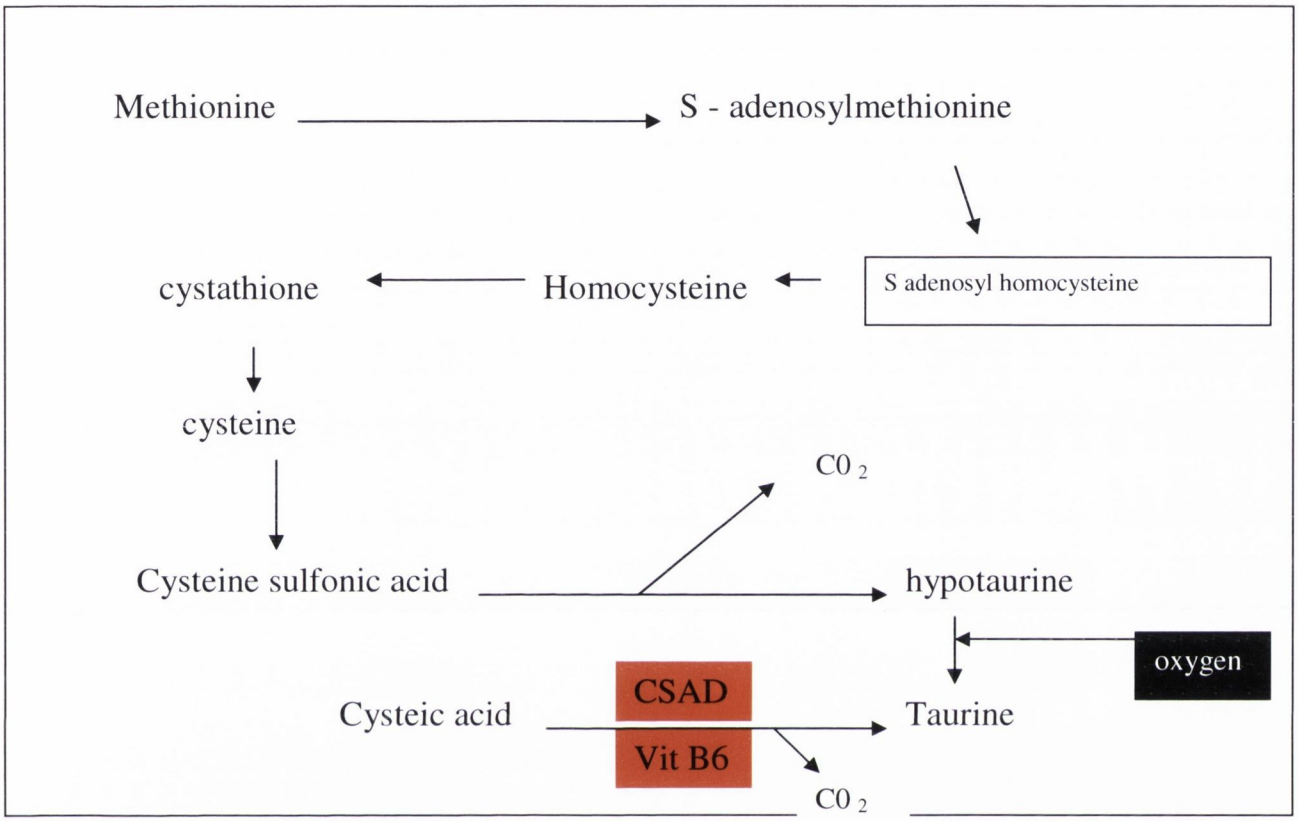


Taurolidine

Taurolidine consists of 2 molecules of taurinamide. In solution it yields taurine. In solution taurolidine is in equilibrium with tauraltam and methyl-taurultam and contains only trace amounts of formaldehyde (< 0.0038%). (Knight et al 1983).



The equilibrium and transformation of taurolidine to taurine in vivo



Taurine biosynthesis from methionine and homocysteine.
CSAD (cysteine sulphinic acid decarboxylase) catalyses the main portion of the conversion of methionine to taurine.
It requires vitamin B6 as a co-enzyme

Method of administration

It has been shown that intraperitoneal and intravenous taurilodine (taurolin) administration produces similar results with regard to distribution in the body and safety (Waser 1985). The approved recommended daily dose range for humans is 2 – 10 grams per day. More than 10,000 patients have received intravenous or intraperitoneal regimes (Waser and Sibler E. 1986). Further studies provide evidence of the safety of intraperitoneally administered taurolidine.

Cytotoxicity

Cyotoxic testing has been performed on taurolin prior to its use on humans. This was performed using using monolayers of tissue culture. Human embryonic lung fibroblasts and two reference strains of human skin fibroblasts were selected as normal, non neoplastic control cell lines. The monolayers were examined by direct light and phase contrast microscopy at 1 hour and 18 hours after exposure for cytotoxicity.

The results suggested that taurolidine and its derivative taurine are non toxic to normal tissues. Subsequent to this taurolin was used in many clinical trials involving humans without side effects.

A 2 % Taurolin solution has been shown to be non toxic to the middle ear mucosa and the inner ear (Handrock and Mathews 1985).

In one study 2% taurolin was perfused through the eardrum of guinea pigs at a rate of 0.21 ml per minute and then rinsed with physiological saline. The auditory thresholds of the guinea pigs were checked daily up to day 14. The amplitudes and latency times of responses showed no change from their original values.

Taurolidine and its metabolites (except taurine) cannot cross the blood brain barrier as shown by autoradiographic studies (Steinbach-Lebbinn 1982).

Taurine however crosses the blood brain barrier via the sodium and chloride ion dependent carrier sysyem (Kang 2000), (Benrabh et al. 1995).

Further evidence that taurine crosses the blood brain barrier and that the rate is dependant on plasma levels until saturation is reached has been shown (Stummer et al. 1995). Therefore the only metabolite of consequence to enter the perilymph of the inner ear via the cerebrospinal fluid is taurine.

The Ear

Taurine entry into the perilymph by passive diffusion from the blood has also been established (Angelini et al 1998). It is known to be the most abundant free amino acid in the inner ear although its exact function is unknown. Studies have shown that localisation of taurine in the organ of Corti is consistent with taurine being involved in the osmotic equilibrium of the normal organ of Corti and perhaps in the restructuration of the pathological organ of Corti. (Horner and Auousseau 1997). Evidence points to taurine having no role as a neoromodulatory amino acid in the inner ear (Usami and ottersen 1995).

In the cochlea a functional study examining auditory evoked brainstem responses in guinea pigs after intraperitoneal taurine administration suggested that taurine actually increases aminoglycoside toxicity at least in the concentrations used by the study (100 mg/ kg/ day for 21 days). This potentiation of ototoxicity is believed to be due to its effect of decreasing the ammount of extracellular calcium, possibly by simple chelation. This allows increased binding and uptake into hair cells. It may also stimulate uptake into hair cells. The study concluded however that any changes in calcium concentration in the inner ear due to taurine are reversible and in the absence of this specific ototoxic insult no cochlear damage would occur (Kay and Davies 1990).

A similar study in mice showed that taurine (N-Chlorotaurine) infusion into the middle ear is well tolerated without adverse effects (Neher et al 2001).

Interestingly a study (Reissner at al 1994) using a 2% solution of taurolin instilled into the ear to treat purulent otitis media in 10 (human) patients showed that the antibacterial effects of taurolin eliminated 12 of 13 bacterial species isolated before

the trial.

Of particular interest was the fact that audiometric testing showed no ototoxic side effects in a follow up period of 30 months (Reissner C et al. 1994).

Thus there is conflicting evidence on the ototoxicity of Taurolin.

The effect of taurine on the vestibular function of the inner ear has not been clinically investigated and the ultrastructural changes induced by aminoglycosides in the presence of supplementary taurine as evidenced by transmission electron microscopy has not been investigated either.

Therefore this study examined the effects of taurine supplementation (via administration of taurolin) on the ultrastructural changes in the guinea pig utricle induced by aminoglycoside ototoxicity.

Methods

The aims of this study are

Aim 1. To document the relative extrusion to apoptosis rate as a mode of cell death in gentamicin induced damage.

Aim 2. To assess the reliability of light microscopy in cell counts. This was done by comparing light microscopy specimens (at X 100 magnification) to the same specimens at Transmission electron microscopy with Formvate covered copper grids.

Aim 3. To document the light microscopic and ultrastructural degenerative and regenerative changes in the guinea pig utricle at 1, 2 and 4 weeks following gentamicin exposure by measuring the relative supporting cell : hair cell ratio changes. The hair cell counts included not only regenerating hair cells identified but also hair cells which were in a state of degeneration, ranging from moderate to severe.

Aim 4. To identify regenerating hair cells.

Aim 5. To identify type 1 and type 2 regenerating hair cell numbers at 1, 2 and 4 weeks post gentamicin damage.

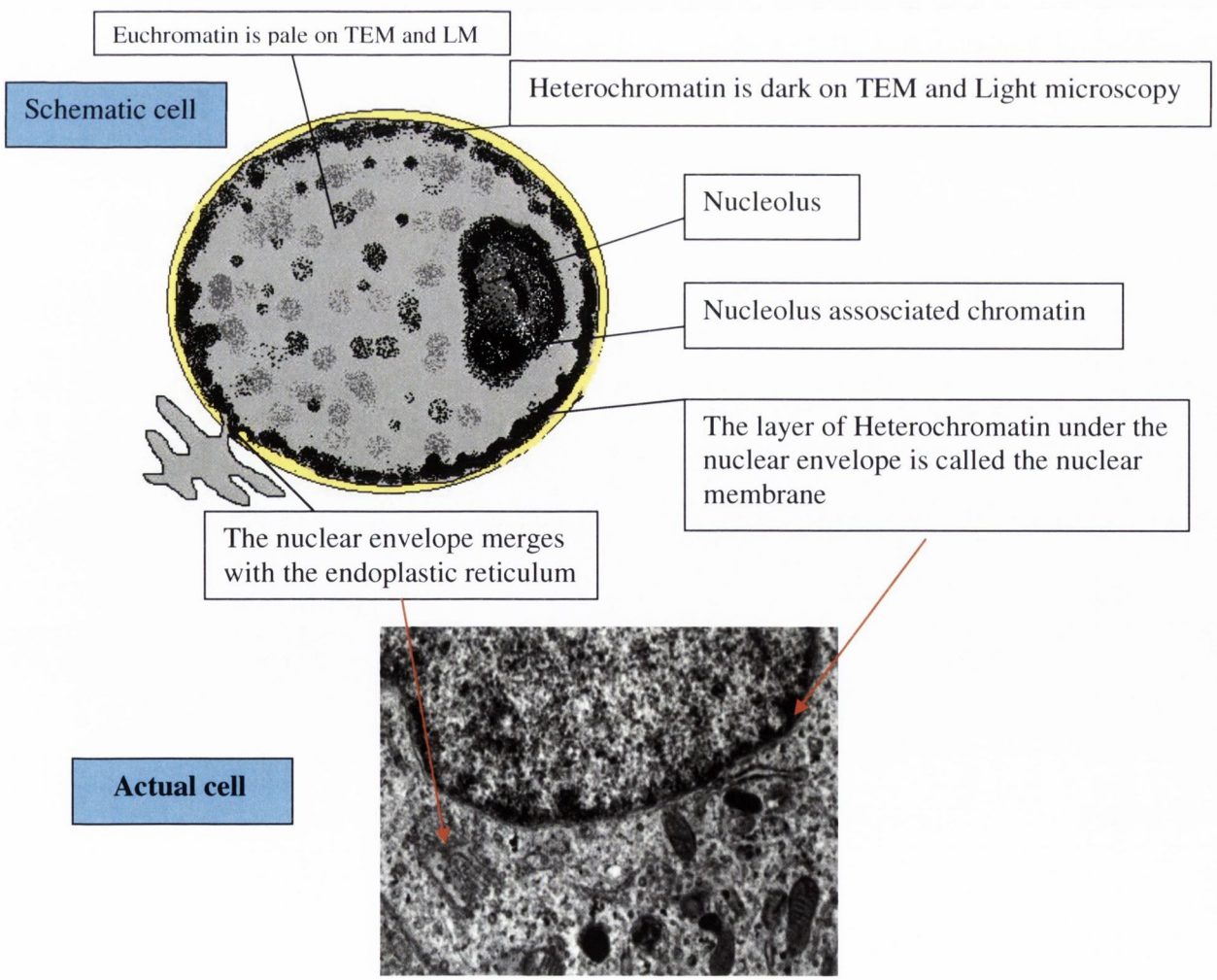
Aim 6. To identify the source of regenerating hair cells by identifying supporting cell nuclear changes or activity around any regenerating hair cells.

An important facet of this aim is to identify supporting cell mitosis at Light Microscopy and Transmission Electron Microscopy. In order to appreciate the changes we need to firstly highlight features of the nucleus of a cell and also the features which indicate that a cell is dividing.

By doing so and understanding these changes any TEM evidence of dividing supporting cells could be identified.

The nucleus contains chromatin which is coiled strands of DNA bound to proteins called histones.

Identifiable features of the nucleus of a cell at TEM



The term ***'Mitotic Figure'*** is used as a general term to describe features seen at Light Microscopy and Transmission Electron Microscopy when a cell is undergoing mitosis.

The term covers features such as

a. Nuclear envelope dissolving.

b. The presence of identifiable chromosomes.

c. Spindle fibres forming from the centrioles to chromosomes.

d. Changes in the shape of a cell.

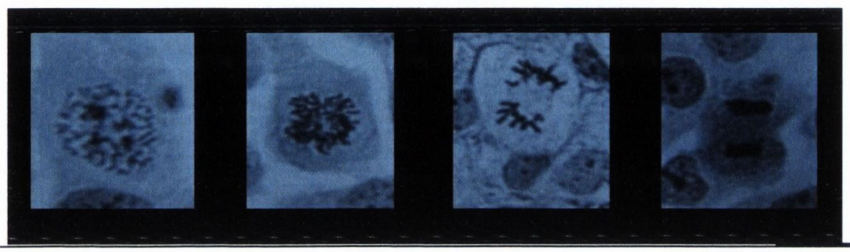
In many cases a cell changes shape (Eg. 'rounding')

e. In some cases changes in the position of the cell.

In simple epithelia the cell rounds, apparently moves towards the surface losing its connection with the basal lamina (Leeson, Leeson, Papano 1985).

f. The classic appearance of the four phases of cell division.

The phases of propahse, metaphase, telophase and anaphase are dynamic and continuous but are subdivided to aid identification.



Photos of light micrographs showing from left to right prophase, metaphase, anaphase and telophase. Reproduced with kind permission from Appleton and Lang.

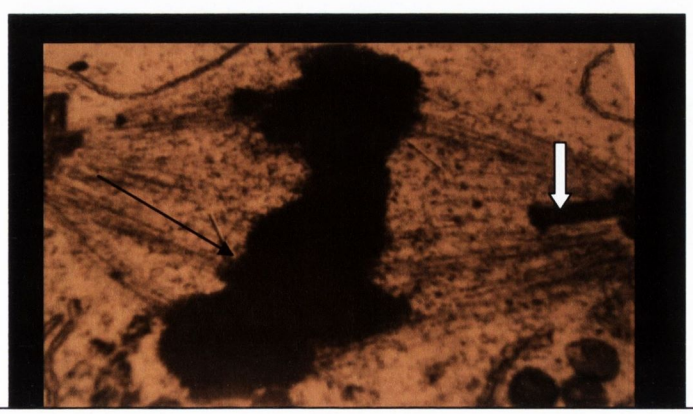
In **Prophase** the chromatin condenses and the chromosomes become dark and short.

The two halves of each chromosome are attached at the centromere.

The nuclear membrane dissolves, the nucleolus disappears and the chromosomes become visible.

In **Metaphase** the chromosomes arrange themselves at the equator.

Under TEM microtubules running from the centrioles at each pole can be seen running to the chromosomes.



TEM taken at Metaphase showing spindles (Microtubules) running from the centrioles (white arrow) to the chromosomes at the equator (Black arrow). Reproduced with kind permission from Appleton and Lang.

In **Anaphase** the cell splits with each copy receiving a set of chromosomes.

In **Telophase** the chromatin condenses to chromosomes, the nucleolus reforms as does the nuclear envelope. The centriole also duplicates.

Aim 7. To investigate whether intraperitoneal Taurolin ® is either cytoprotective or potentiates aminoglycoside induced damage to the mammalian peripheral vestibular system.

Aim 8. Finally a new technique for atraumatic removal of the utricle is described. Any damage induced accidentally by microforceps or other instruments is eliminated and thus any imperfections in the collected specimens avoided.

Taranstymanic gentamicin injection.

Gentamicin was chosen as the lesioning agent as it is known to be a primarily vestibulotoxic agent.

The right side was the side chosen for treatment with gentamicin in all of the animals.

Commercially available gentamicin sulphate 2mg per ml was used. This preparation contains preservatives which have been shown to be non toxic to the inner ear of guinea pigs and humans.

Duncan Hartlett guinea pigs of both sexes and aged 8 weeks at the time of injection were used in this study.

The weights of the individual guinea pigs were assessed using an electric scales.

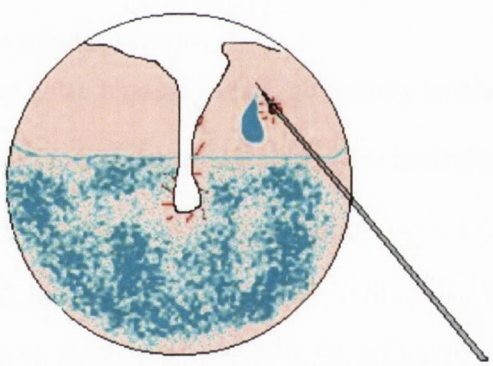
The animals were anaesthetised with intramuscular xylazine (Rompum ® 0.2ml/kg) and ketamine (Ketalar ® 1.5 ml/kg) injected into the thigh of the hind leg and the hair around the right ear was shaved to facilitate access to the tympanic membrane.

An operating microscope (magnification X 40) and a dental needle was used to inject the gentamicin into the middle ear cavity.

The middle ear cavity was filled via a small injection site through the antero-superior part of the tympanic membrane to prevent escape of the gentamicin.

Specific care was taken to avoid tearing the tympanic membrane and to avoid ossicular damage.

The injection was performed over fifteen seconds to prevent mechanical injury to the middle and inner ears.



Injection of the gentamicin through the tympanic membrane

The guinea pigs were placed on their left side with the head tilted in the upright direction during recovery from anaesthesia to further prevent escape of gentamicin. During this time their bodies were covered with straw and placed under a heating lamp for the half hour or so that it took to recover from the anaesthetic.

The animals were observed intermittently for a further four hours upon recovery.

Gentamicin can be delivered to the middle ear cavity by sustained release, by a once off injection or series of injections (Hoffer et al. 2001). The drug is absorbed from the middle ear through the thin membrane covering the round window.

The concentration of gentamicin in the spiral ganglion and the hair cells of the inner ear is significantly greater in the transtympanic method of administration than if the drug was introduced systemically (Hoffer et al. 2001).

With transtympanic administration there is a high peak at 24 hours and a rapid falloff with total elimination from the middle ear in 48 hours (Hoffer et al. 2001).

In the sustained release administration there is a long plateau with a peak approximately 1/3 of the size of the transtympanic method of administration.

The reliability of sustained release administration decreases functional and morphological variation between animals but while perilymph kinetics differ, the results are the same from a functional and morphological viewpoint.

(Hoffer et al. 2001). Therefore for ease of administration and in order to limit stress to the guinea pigs, a once off dose of transtympanic gentamicin injection was preferred.

Taurine injections

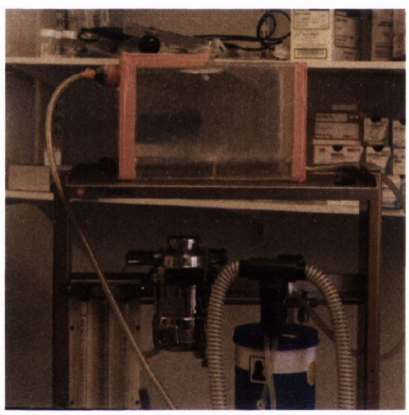
The taurolin (200 mg/kg body weight) solution was injected intraperitoneally under sterile conditions one hour before gentamicin injection to ensure that it was in the plasma and hence the cerebrospinal fluid and perilymph of the inner ear. This dose had been injected intraperitoneally in mice before so the same dose was used in the guinea pigs. Thereafter it was injected intraperitoneally at the same dose every third day except day 13 until the guinea pigs were harvested. This meant that in total four doses were given to each guinea pig.

Five animals were treated in this fashion with the right ear being the side to receive the gentamicin and the left serving as a control.

The guinea pigs were harvested at 14 days post gentamicin injection.

Removal of the utricle

The Duncan Hartlet guinea pigs aged 8 weeks at the time of gentamicin administration were first anaesthetised with a halothane / oxygen mixture via a specially prepared chamber in order to avoid the discomfort of the injection of intramuscular xylasine (0.2 ml/kg) and ketamine (1.5 ml/kg).

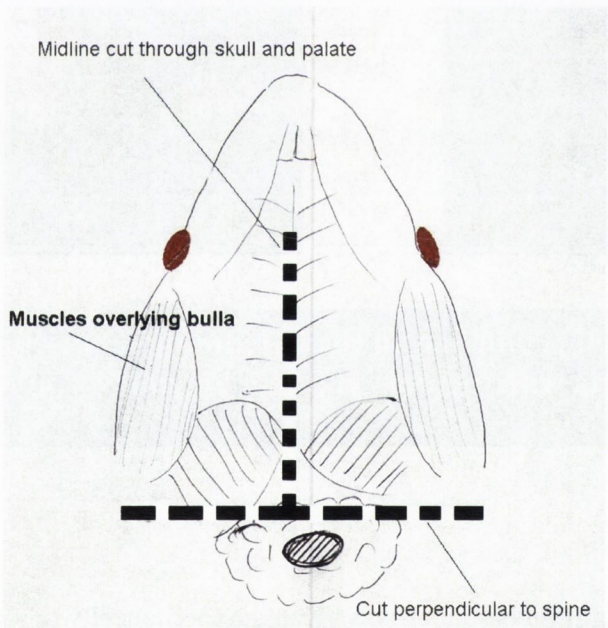




The anaesthetic chamber on the left and intramuscular anaesthetic agents with pentobarbitone (Sagatal ®) on the right

Then a lethal dose of pentobarbitone was given intraperitoneally to sacrifice the animals under the observation of the animal experts in the research laboratory. This ensured a humane and painless harvesting of the utricles.

Upon repeated demonstration of a loss of the paedal reflex by two observers, the guinea pigs were decapitated. The mandible was removed by cutting the rami with a heavy duty scissors. Next a horizontal cut with a scissors was made perpendicular to the spine to remove any tissue obscuring the foramen magnum. Using the scissors a midline cut was made along the base of the skull continuing through the hard palate. This released the bony restraints to facilitate removal of the bullae.

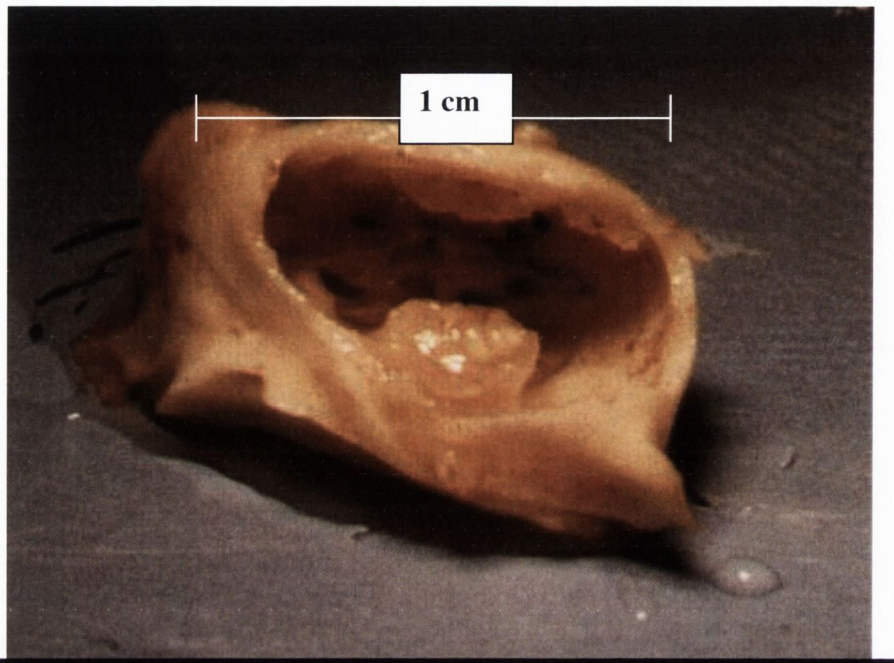


To establish a system the right and left bullae were harvested in a rotating manner. This meant that if the right bulla was harvested first on a particular guinea pig, then the left was harvested first on the next.

The bullae shelled out in a clean manner from the skull allowing complete removal of the middle and inner ears from the animals without damage. Immediately upon removal the convex side of the bulla was entered with the aid of a scissors and the bony cochlea exposed. Immediately following this, the basal turn of the cochlea was removed with a forceps and several drops of 2.5 % glutaraldehyde with phosphate buffer were instilled into the specimen under the microscope. This was to ensure that no air bubbles were present in the specimen. The bulla was than immersed in the same solution for preservation.

The whole procedure from decapitation to immersion of both of the prepared bullae in gluteraldehyde / phosphate buffer took less than two and a half minutes in most cases and less than three minutes in all cases.

This ensured that no hypoxic damage would interfere with LM or TEM examination of the utricles.



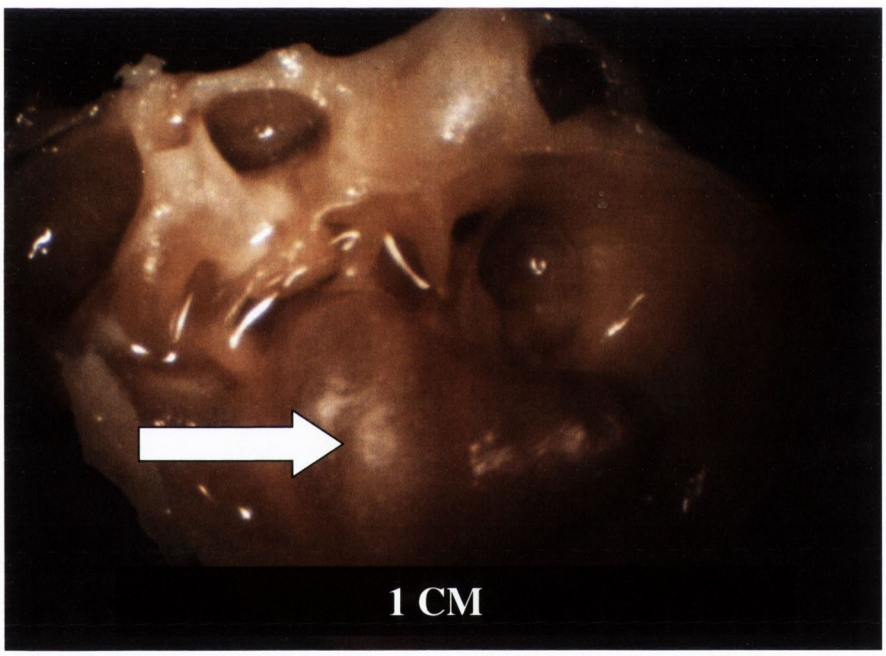
Removed bulla with its convex surface removed to show the cochlea

Access to the utricle

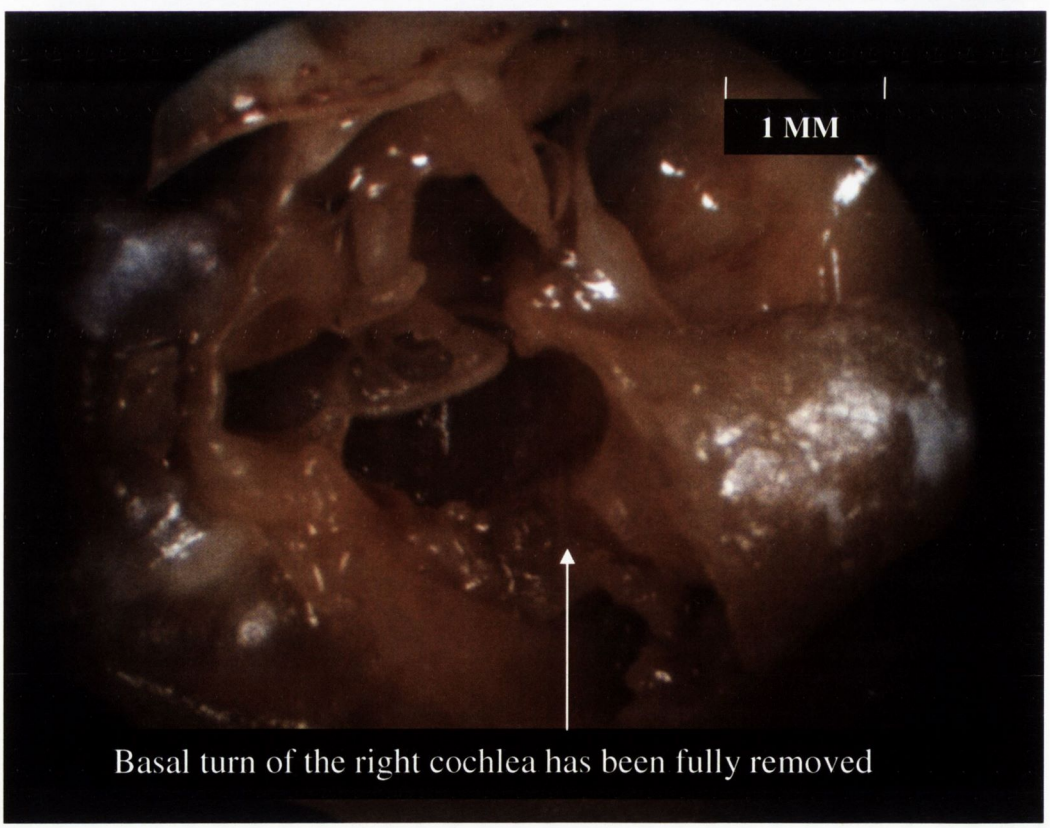
The utricle is found in the vestibule. It can usually be identified by the fact that it is white and heart shaped or kidney shaped whereas the saccule is comma shaped.

The right utricle faces left and visa versa. Both the basal turn of the cochlea and the stapes footplate allow access to the vestibule once removed.

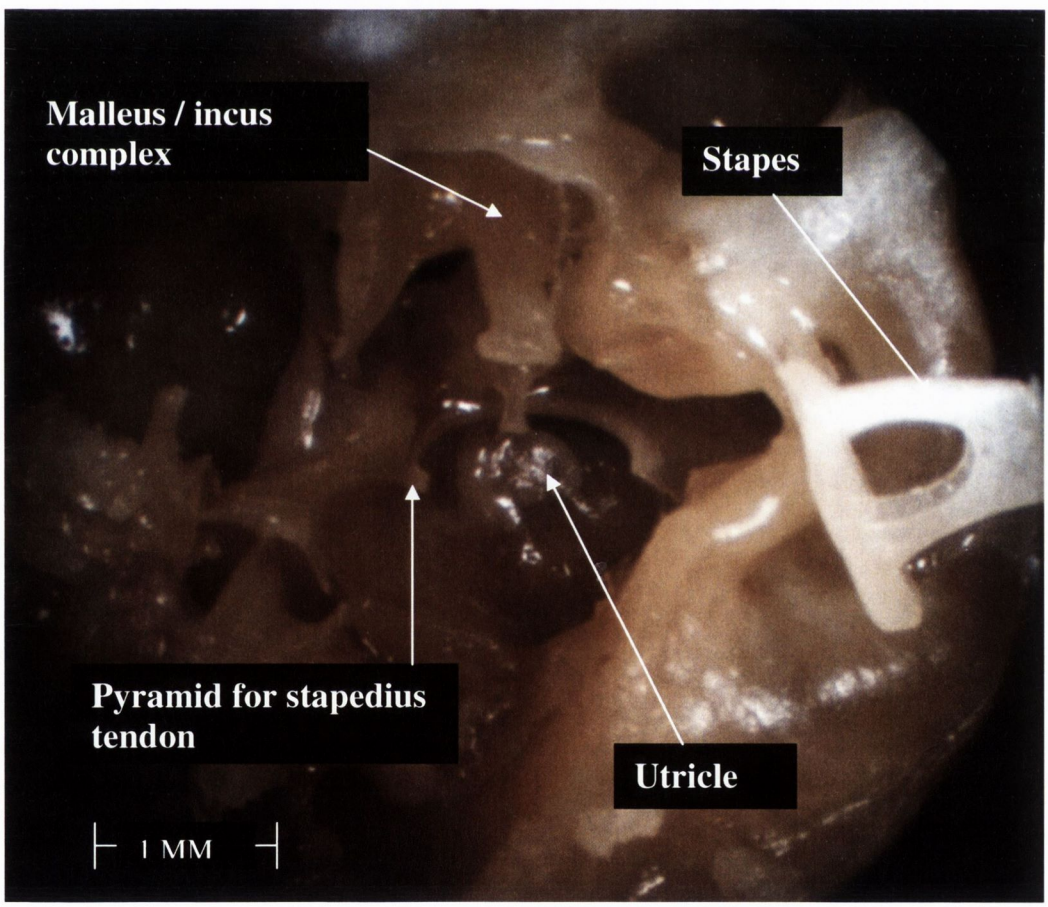
During harvesting of the bullae the basal turn of the cochlea is partially removed (white arrow).



With the time constraints on tissue immersion in gluteraldehyde / phosphate buffer solution it is not possible to carefully dissect the whole basal turn and any attempt to remove the basal turn in its entirety would leave the utricle susceptible to hypoxic damage. Therefore at a later stage during dissection (in gluteraldehyde / phosphate buffer solution) the remaining bony strut of the basal turn was removed. The utricle is now visible in the solution but not without the solution as diffraction allows identification below the stapes footplate.



The malleus / incus assembly (which is fused in rodents) is dislocated from the stapes head and the stapedius tendon is cut.

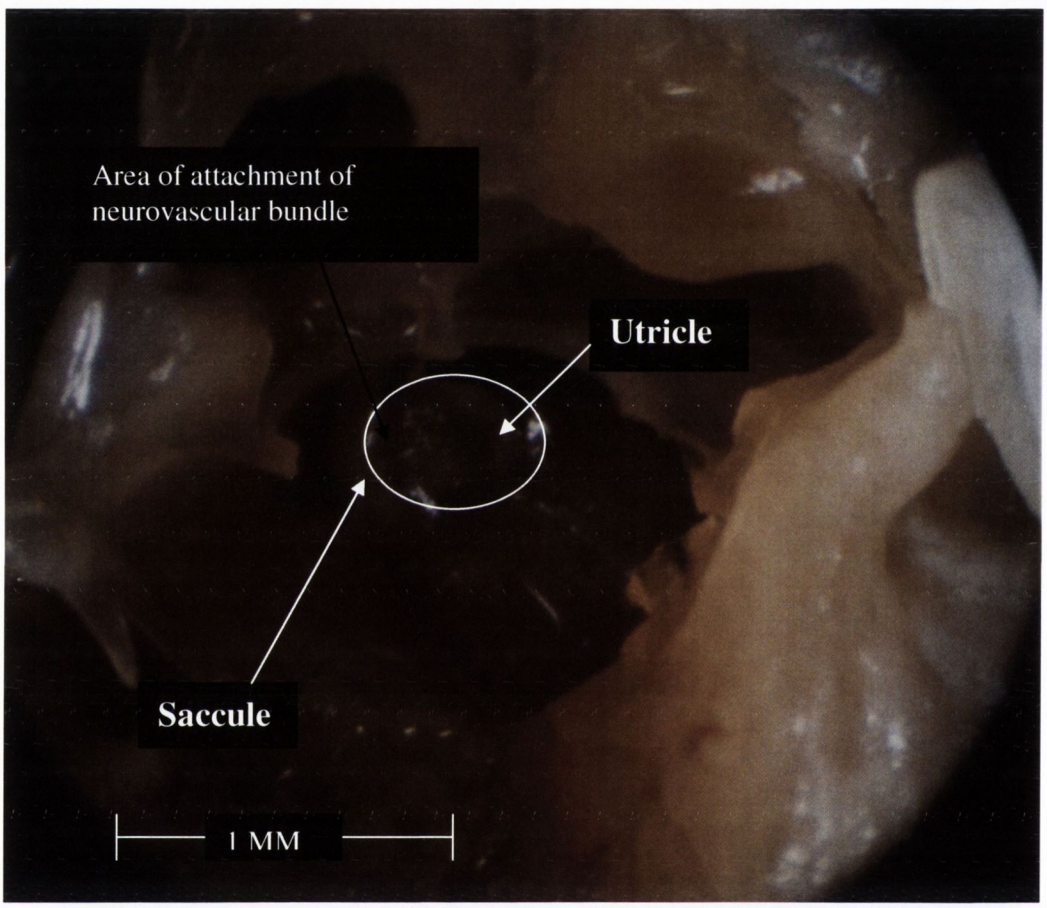




Removal of the stapes

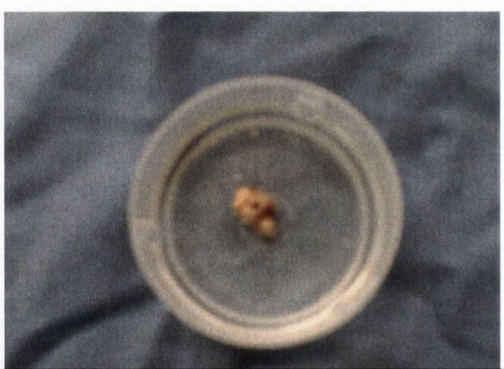
The malleus / incus complex and the pyramid, a bony attachment for the stapedius tendon are removed to facilitate access to and removal of the utricle.





Using two bent dental needles, the utricle is freed of its membranous attachments and then is removed from its attachments to the neurovascular bundle.

Finally the bulla was now inverted in a perfectly clear glass petri dish containing gluteraldehyde / phosphate buffer solution and the utricle, when properly freed from its membranous attachments falls gently to the bottom of the dish where it is removed with the aid of the gentle action of a large bore suction pipette. This method of atraumatic removal has not been previously described.



Upturned bulla immersed in glutaraldehyde / buffer solution to release the utricle

Ten guinea pigs killed humanely for utricle harvesting at 1 week after gentamicin injection.

Ten guinea pigs killed humanely for utricle harvesting at 2 weeks after gentamicin injection.

Ten guinea pigs killed humanely for utricle harvesting at 4 weeks after gentamicin injection.

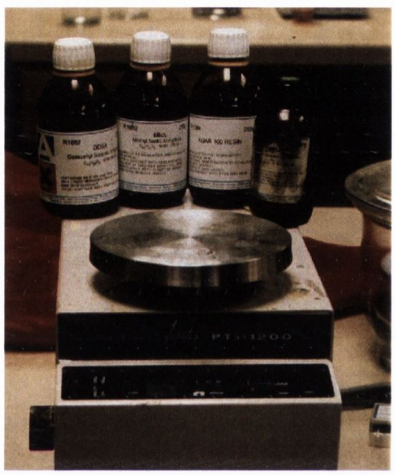
Five guinea pigs underwent transtympanic gentamicin injection one hour after intraperitoneal injection of Taurolin ®. They had intraperitoneal taurolin injections every third day except day 13. They were humanely killed at 2 weeks after the gentamicin injection for utricle harvesting.

The harvesting of utricles at 1, 2 and 4 weeks post gentamicin administration meant that the damage caused by the gentamicin was at its greatest level and previous studies have shown that the density of regenerating hair cells is greatest at 4 weeks post gentamicin exposure (Forge et al. 1993, Quint 1996, McConn-Walsh 2000). Therefore maximum examination of degeneration and regeneration processes could be studied.

Preparation of tissue for transmission electron microscopy

Step 1.

Epon is a resin which is made up from its components and allowed to mix on an electric mixer.



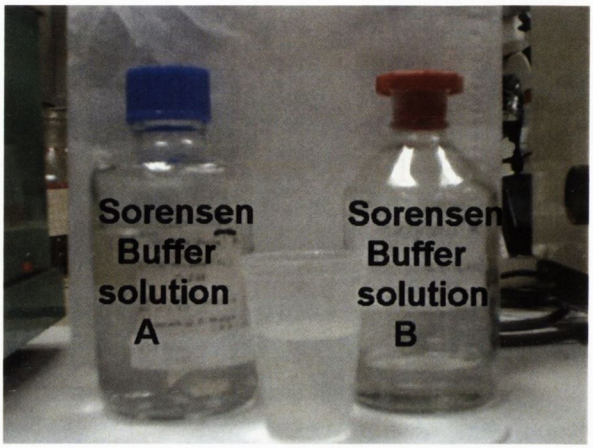
Step 2

The tissue is stored in gluteraldehyde / buffer solution and must not be allowed to dry as this causes distortion.



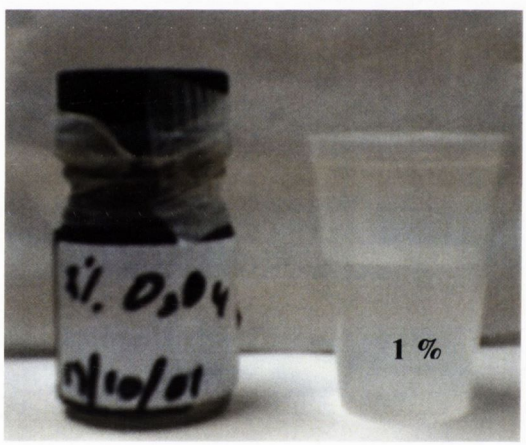
Step 3

The tissue is removed from the gluteraldehyde and placed in a phosphate buffer solution for 10 minutes. This buffer solution is made from the addition of Sorenson A and B solutions.



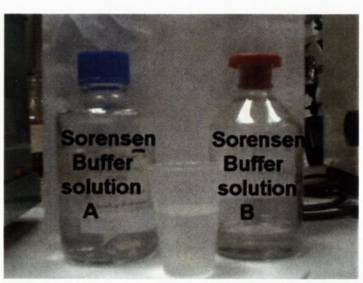
Step 4

The tissue is then placed in a 1% solution of Osmium tetroxide for 1 hour. Osmium is a potent tissue fixative and is used in the fume cupboard only.



Step 5

The tissue is placed in phosphate buffer solution for a further 10 minutes at this stage.



Step 6

The tissue is now placed into a 70 % ethanol solution for 10 minutes to dehydrate it.

Step 7

Step 5 is repeated

Step 8

The tissue is now placed into a 90 % ethanol solution for 10 minutes to dehydrate it.

Step 9

Step 5 is repeated.

Step 10

The tissue is now placed into a 100 % ethanol solution for 20 minutes to dehydrate it.

Step 11

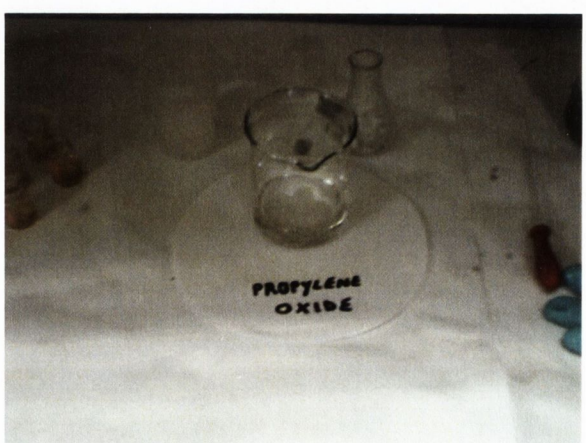
Step 5 is repeated.

Step 12

Step 10 is repeated.

Step 13

The tissue is placed into a propylene oxide solution for 15 minutes. Propylene oxide is an organic solvent and must be used in a fume cupboard.

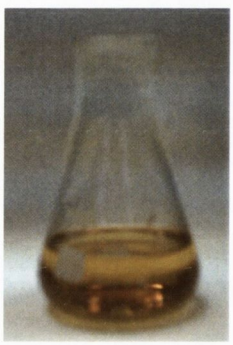


Stage 14

The tissue is placed into a propylene oxide solution for a further 15 minutes.

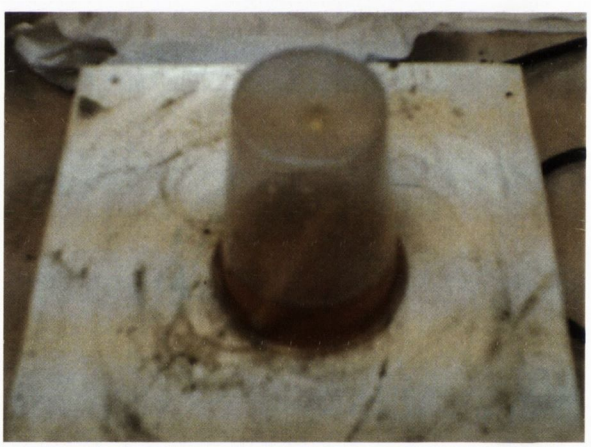
Stage 15.

The tissue is placed into a solution made from equal parts Epon and propylene oxide solution for one hour.



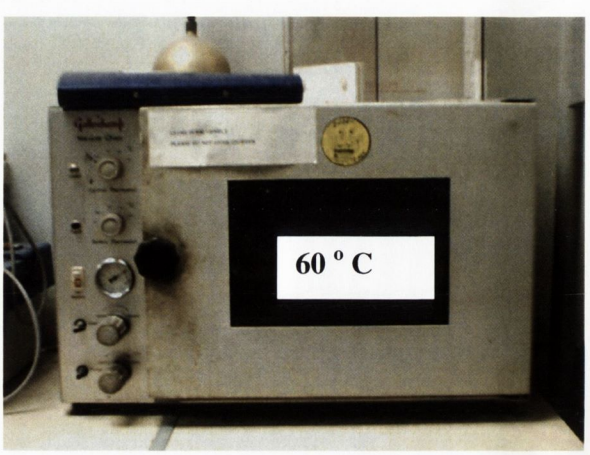
Step 16

The tissue is now placed into Epon and incubated at 37 degrees centigrade for 2 hours to evaporate the propylene oxide and allow the resin to fully replace it at a molecular level. It is therefore important to have the solution uncovered.

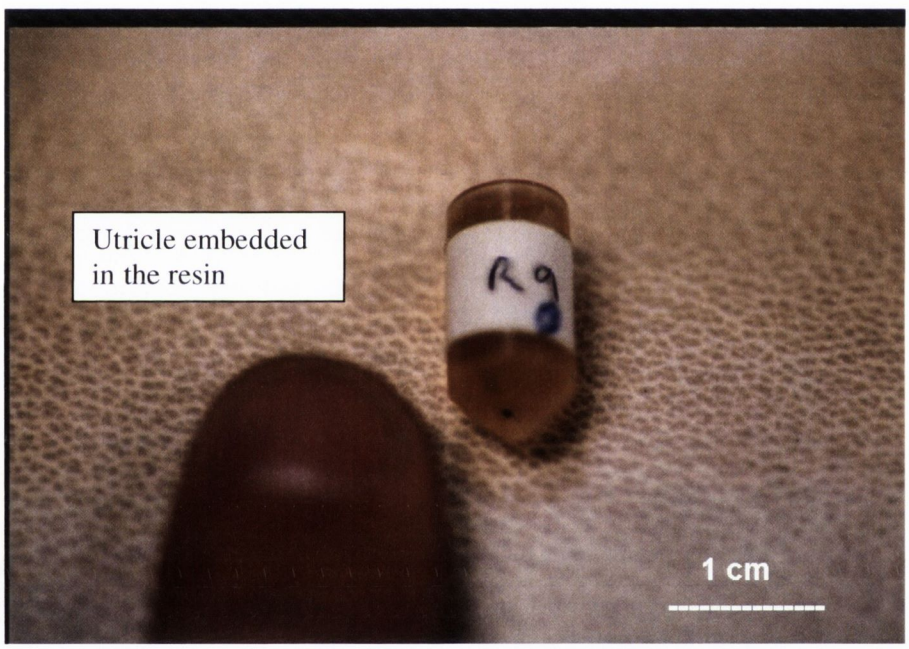


Step 17

Finally the tissue is placed in Epon and incubated at 60 Degrees Centigrade overnight.



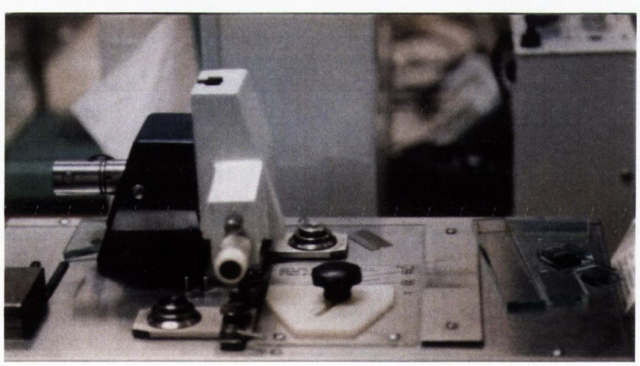
The resin is now hard enough to facilitate ultramicrotomy sectioning and staining.

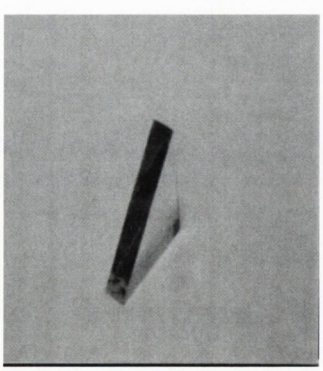


Making the glass knives

This task was performed using a glass knife maker.

First the pre-prepared glass rods are placed in position on the device and locked in place. The glass is scored with a tungsten tip and then carefully fractured along the score line to form a square shaped piece of glass. The glass fractures with two identifiable dimples on one side of the square. These dimples are placed at the lower left quadrant and the square piece of glass further scored and fractured to produce two knives each with one cutting surface. The fracture line makes a knife with a sharp edge on the left hand side of the knife surface and it is with this edge that the specimen should be cut.

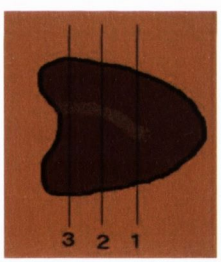


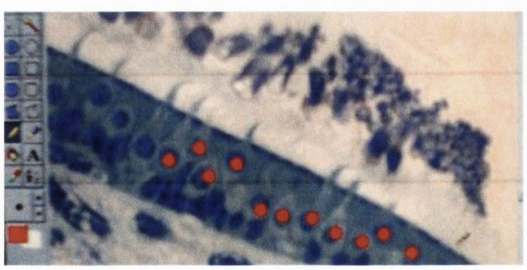


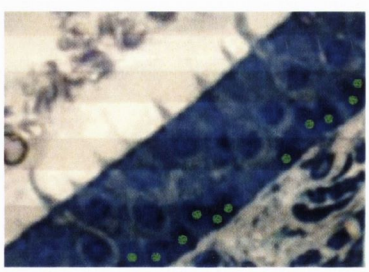
Shaping the specimens

The specimen is orientated in the correct plane in the Epon before cutting light and electron microscopy specimens.

This required the utricle to be approached in the horizontal plane perpendicular to the striola. The utricle was sampled in three separate locations along its length and at each location one section was examined by light microscopy and one by electron microscopy. The ideal and useable specimen yielded a cross section of the utricle with clearly identifiable supporting cells and hair cells cut as close as was possible to the perpendicular plane to the striola.







Representation of how the utricles were sectioned (above) and how the specimens were examined on computer for cell counts (below). The hair cells nuclei were marked with a red dot. The supporting cell nuclei were marked with a green dot.

The epoxy resin (Epon) was shaped as a pyramid with the top removed in order to facilitate collection of the specimens at ultramicrotomy.

The ultramicrotome



The diamond knife

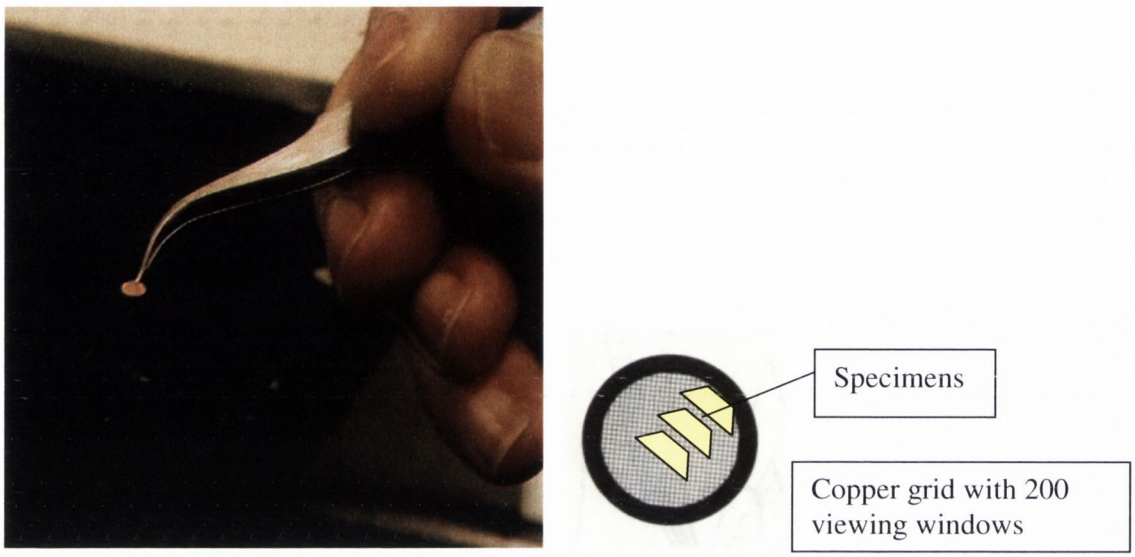


Firstly a light microscopy slide was prepared using a 2 micrometer section and examined under a Leica light microscope. Once the layers of the utricle could be clearly identified, the ultramicrotome was used to cut sections of the same spot for the electron microscope.



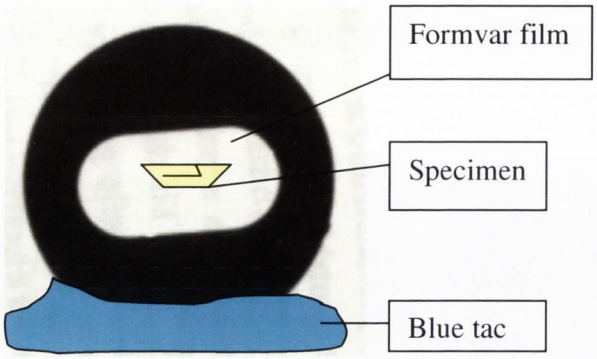
The light microscopy slide is stained with toludine blue for one minute and heated gently to fix the specimen, a process called annealing.

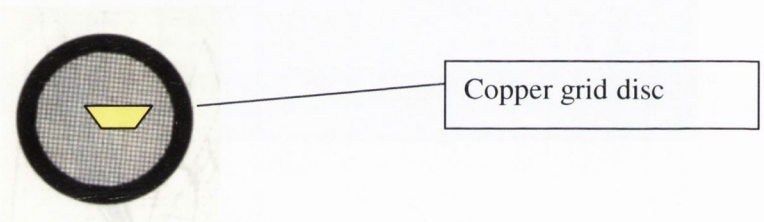
Collecting specimens for transmission electron microscopy staining

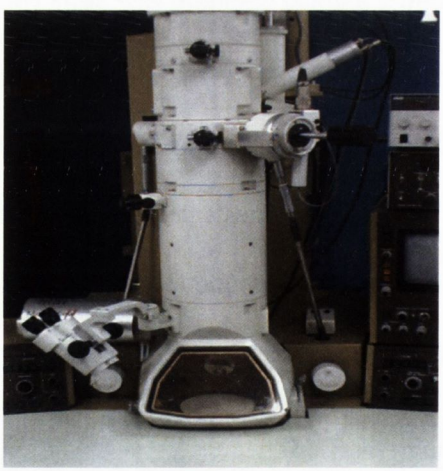


Preparation of the Formvar grids

Formvar is a chemical consisting of a 0.5 % solution of formvate in chloroform. A glass slide is dipped into the formvar after polishing the slide with oil to produce a perfectly smooth surface. The formvar dries and is scored on the glass slide. It is then placed into a bowl of distilled water where the formvar floats on the surface as an ultrathin clear film. The copper grids are placed onto this film. Next the film is collected with the aid of a bioplastic film and the grids cut out with a blade. The specimens are collected, stained in the same way and dried upright with the aid of blue tac to produce a fine window which allows specimens to be examined without fear of a view obstructed by grid bars at transmission electron microscopy.







The specimen can now be examined by Transmission electron microscopy

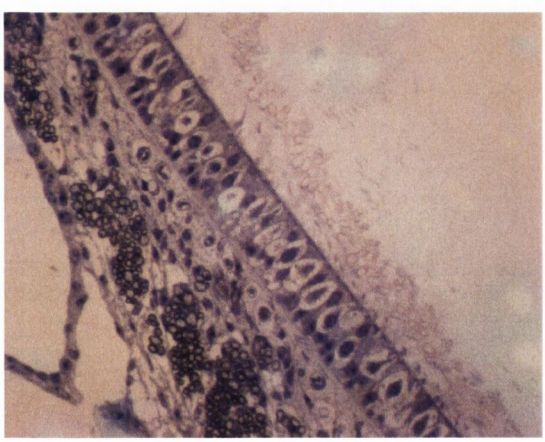
Results

Assessment of the major method of cell death after Aminoglycoside induced damage

Light microscopy examination and subsequent TEM examination showed that most cells which died underwent death by apoptosis. Definite extrusion was only identified clearly in thirteen cells despite close examination of all specimens at Formvar TEM.

It is possible that one example of extrusion was identified at light microscopy. Therefore using the strict criterion of clear evidence of extrusion occurring at TEM it appears that over 99.99 % of cell death after gentamicin induced toxicity occurs by apoptosis. Note that the supporting cells remained undamaged.

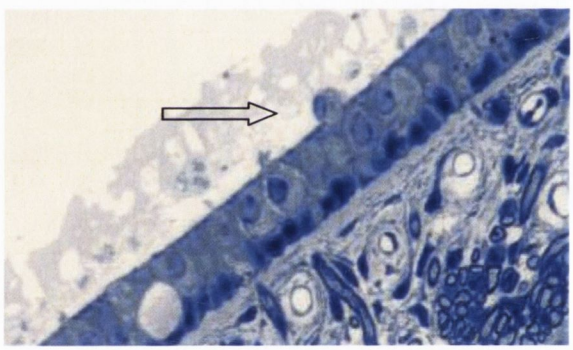
Supporting cells have also shown to be unaffected by gentamicin in cultured vestibular cell experiments (Forge A. Li L. 2000).



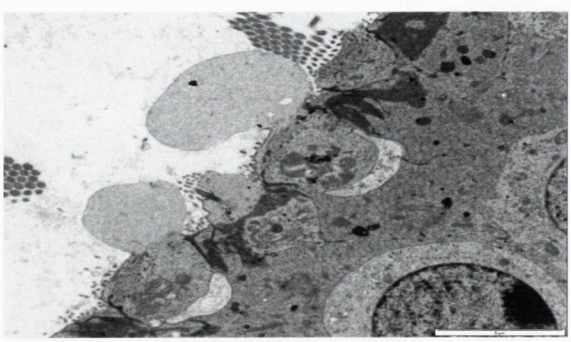
Light microscopy specimen showing widespread apoptosis of hair cells (X 100).



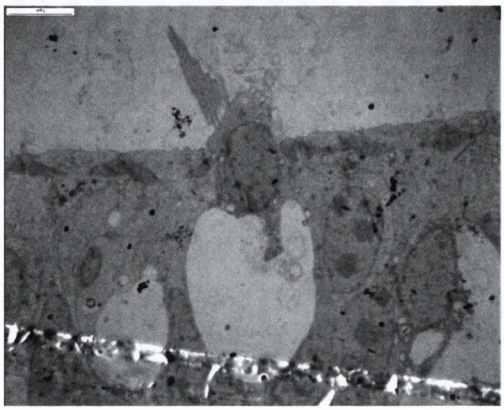
The same specimen examined at TEM showing apoptosis of three hair cells (Black arrow). Note that the supporting cells remain undamaged and that their nuclei are unusually high in the macula (Red arrow) (x 2500).



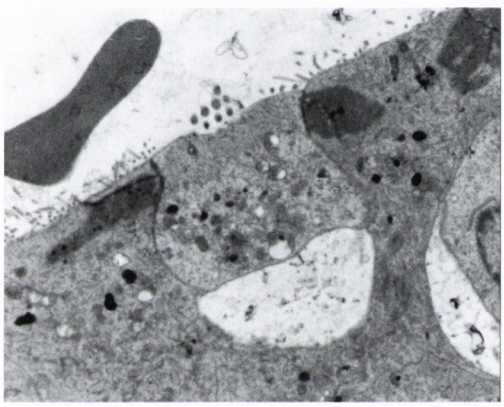
Light microscopy example showing extrusion of a gentamicin damaged hair cell (x 100).



Examples of extrusion identified at TEM (X 3000)



TEM image showing a combination of extrusion and apoptosis in an irreparably damaged hair cell (X 2,500)



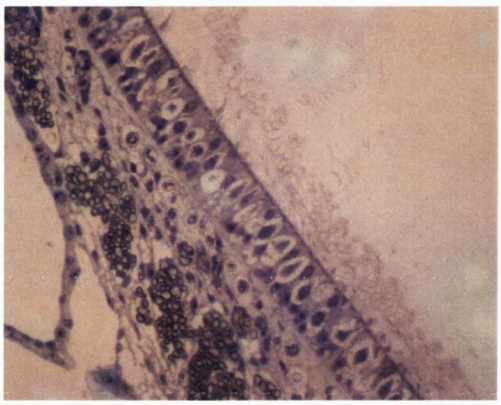
TEM showing an apoptotic hair cell which is undergoing extrusion (x 3000)

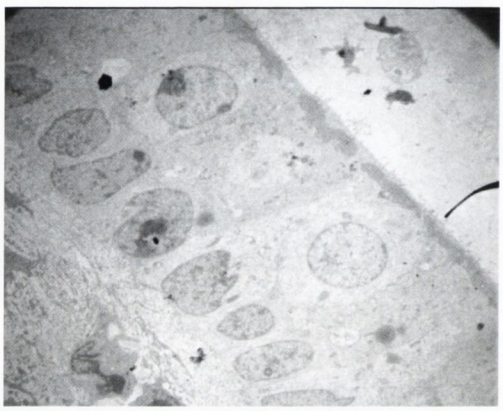
This study demonstrates that apoptosis is by far the major cause of cell death.

The study does show however that extrusion does occur and that a combination of extrusion and apoptosis also occurs.

It also confirms that supporting cells are not affected by gentamicin toxicity in the doses used in this study.

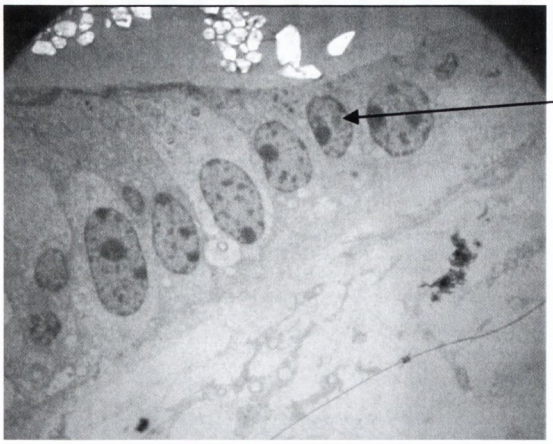
Formvar and Light microscopy count correspondence





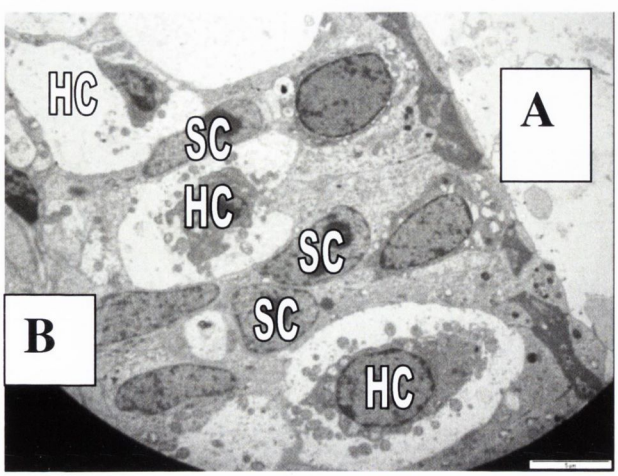
The supporting cell counts and the hair cell counts of the same specimens viewed at light microscopy and Formvar grid transmission electron microscopy corresponded although not precisely. On fifteen single sections of the utricles examined there was a discrepancy between the counts of supporting cells and hair cells at light microscopy and Formvar TEM counts. This was never more than two cells indicating that the light microscopy is sufficient for cell counts.
It was found however that Formvar has two specific advantages.

1. The area where cell counts begin is where the epithelial surface of the membrane which attaches to the macula becomes the macula. This is where the first hair cell is identified. This is much easier to identify under TEM especially where there has been damage to the apical stereocillia. This accounted for the rare discrepancies between the light microscopy and Formvar counts.



The point is illustrated here.
This TEM with Formvar shows a hair cell at the far extremity of the macula. Such a hair cell which when damaged has no identifiable stereocillia may well be missed at light microscopy causing a subtle misreading in hair cell / supporting cell counts. Note the overlying otoconia indicating the presence of previous hair cell processes being present underneath. This problem is easier to identify at TEM (Mag x 2,000).

2. When using cell nuclei to identify supporting cells and hair cells, the nuclei of the hair cells are found in the upper 2/3 of the macula. The nuclei of the supporting cells are normally found in the lower 1/3. In gentamicin damaged maculae the supporting cell nuclei are often found in the upper two thirds of the maculae and therefore when relying on nuclear counts at light microscopy supporting cells can mimic hair cells. At Formvar TEM the cell membranes can be accurately followed and hence it is easy to identify whether a nucleus belongs to a distorted supporting cell or an adjacent severely apoptotic hair cell



TEM showing supporting cells and hair cells in a damaged macula.
Note the position of the supporting cell nuclei which could be mistaken for hair cell nuclei in such specimens at light microscopy (TEM X 2,500).

HC = Hair Cell
SC = Supporting Cell
A = Apical end
B = Basal end

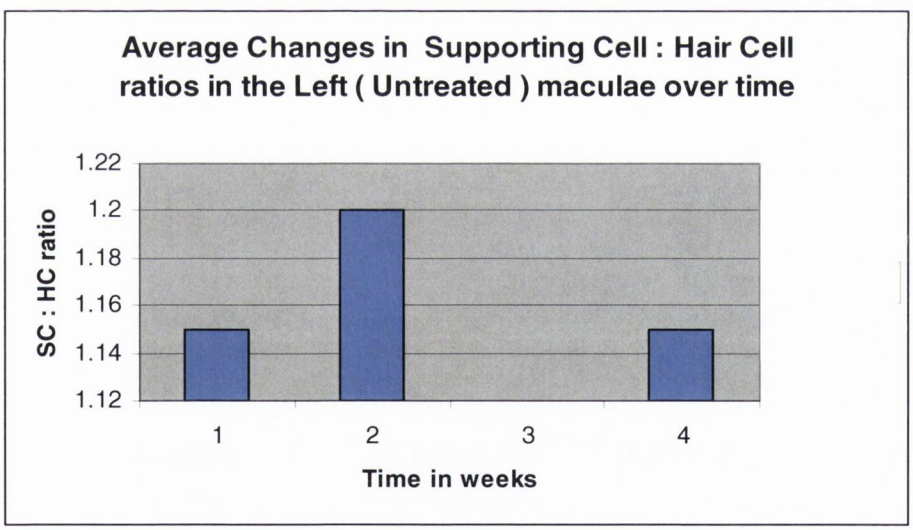
The use of Formvar prepared transmission electron microscopy specimens hence yields the same data albeit slightly more accurate than light microscopy with respect to cell counts. It is therefore the preferred method of examination for this type of study and future studies of this nature. For these reasons the Formvar counts were used to identify supporting cell : hair cell ratios.

Results of supporting cell and hair cell counts over 1 to 4 weeks post gentamicin.

Cell counts were made by counting nuclei for supporting cells and hair cells at Formvar Grid TEM

All of the left maculae from the guinea pigs sacrificed at weeks 1, 2 and 4 were included in the graph and figure below.

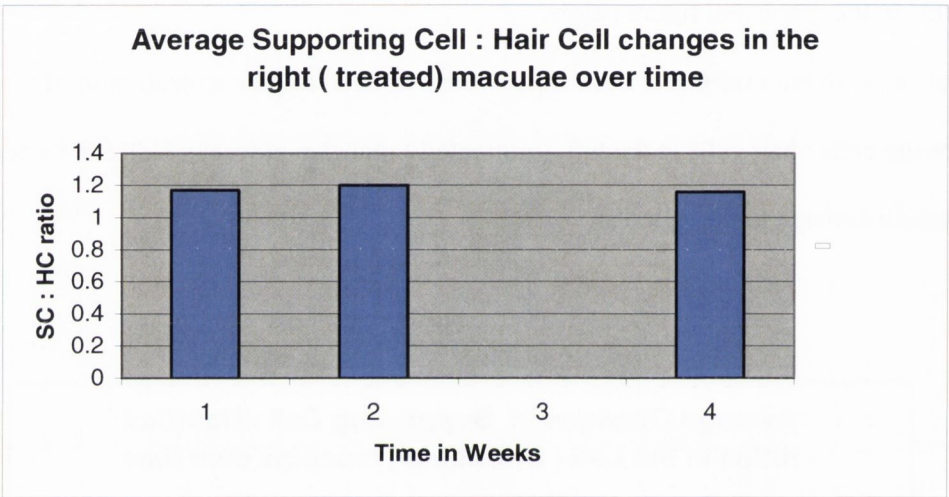
The ratios of all ten maculae at each week were averaged to give a mean ratio of supporting cells : hair cells in the left (untreated) maculae at weeks 1, 2 and 4 after gentamicin damage to the right ear.



Mean SC : HC Ratios Of all 10 untreated maculae	**1.15 :1**	**1.2 : 1**	**1.15 : 1**
	(week 1)	**(Week 2)**	**(Week 4)**

All of the right maculae from the guinea pigs sacrificed at weeks 1, 2 and 4 were included in the graph and figure below.

The ratios of all ten maculae at each week were averaged to give a mean ratio of supporting cells : hair cells in the right (treated) maculae at weeks 1, 2 and 4 after gentamicin damage to the right ear.



Mean Ratios SC : HC	**1.17 : 1 (week 1)**	**1.2 : 1 (week 2)**	**1.16 : 1 (week 4)**

There was no TEM evidence of supporting cell death after exposure to gentamicin toxicity. This is important in interpreting the results.

There was an increase in the supporting cell : hair cell ratio in both maculae at 1 week. This ratio should be 1 : 1.

There was a *relative* increase in the number of supporting cells in both the treated maculae and the untreated maculae the following week, ie 2 weeks post gentamicin induced damage.

The ratio of supporting cells to hair cells was largest at 2 weeks in both the treated and untreated maculae. Interestingly the relative ratios at this time were equal in the treated and untreated maculae.

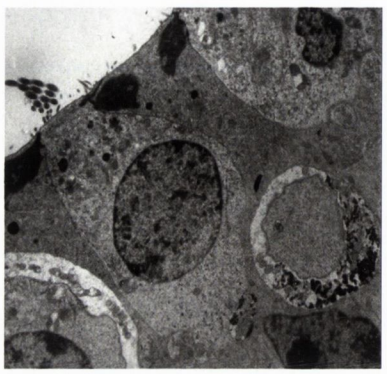
At four weeks the SC : HC ratio remained high in the treated maculae but declined to the same level as was found at 1 week in the untreated maculae.

These results suggest that systemic absorption of gentamicin from the right ears occurred and subsequently caused damage to the left ears.

Hence both the Formvar TEM *counts* and absence of any supporting cell deaths taken together provide evidence to support supporting cell mitosis or supporting cell transdifferentiation as origins to hair cell regeneration in the mammalian inner ear. Due to systemic absorption this occurred in both treated and untreated ears.

Regenerating cells





The total number of regenerating Hair Cells identified at TEM using the **Immature Hair Cell Bundle identification criteria in the treated ears was 87**.

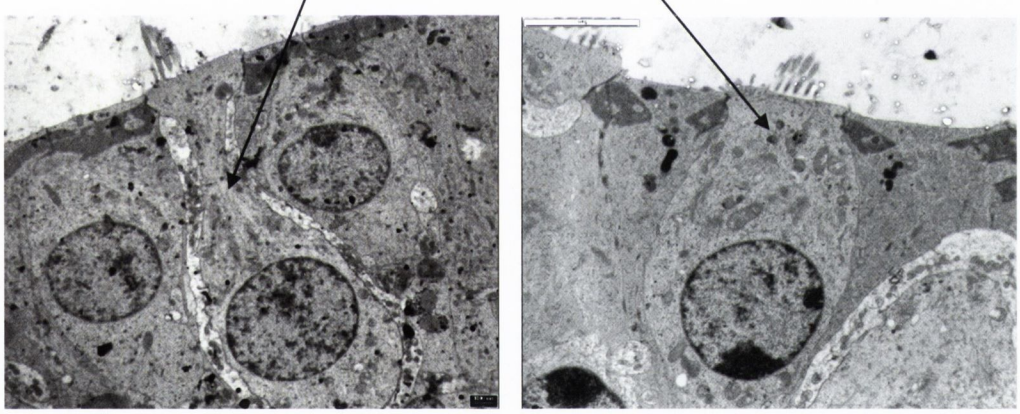
This was from a total of 105 separate sections of the treated maculae (3 x 35). This figure included the Taurolidine treated group.

The total number of regenerating Hair Cells identified at TEM using the **Immature Hair Cell Bundle identification criteria in the untreated ears was 36.** This was from a total of 105 separate sections of the untreated maculae (3 x 35). This figure included the Taurolidine treated group.

Type 1 and 2 counts and differences between Lm and TEM

The accuracy of identifying the total type 1 and 2 regenerating hair cells is vastly improved when examining Formvar TEM specimens. Therefore these Formvar specimens were exclusively used when counting the identifiable type 1 and type 2 regenerating hair cells.

Regenerating type 1 (left) and type 2 (right) Hair Cells
(TEM Mag. X 1,500)



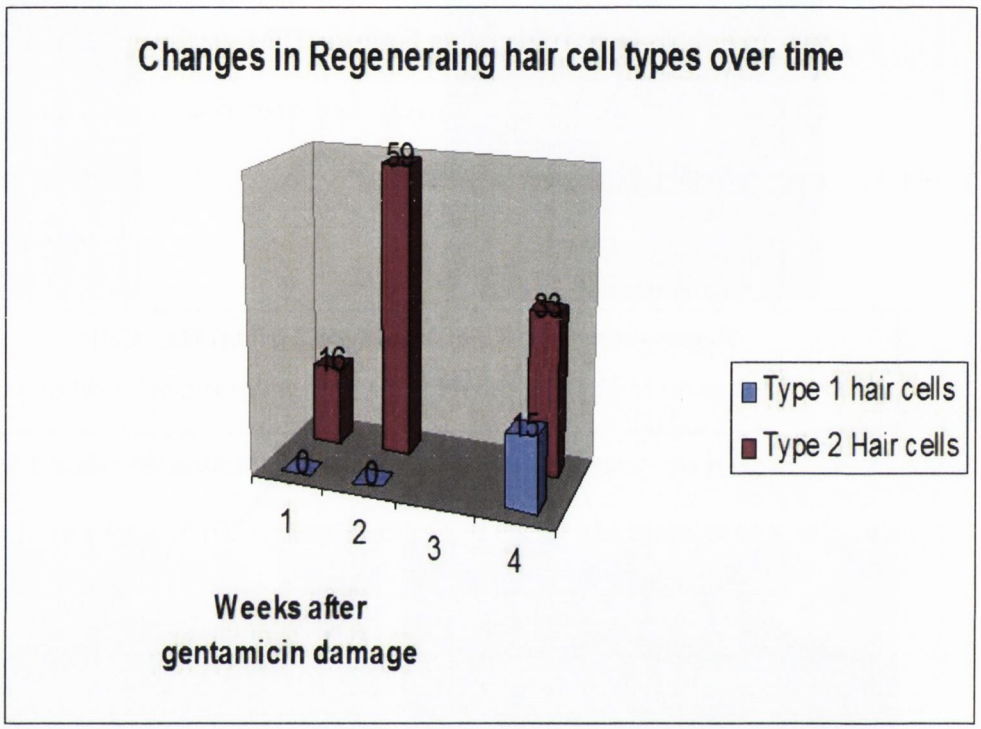
Total number of identified regenerating hair cells was **123**.

15 were identified as regenerating Type 1 hair cells.

108 were identified as Type 2 regenerating hair cells.

All of the type 1 regenerating hair cells were identified only at 4 weeks post gentamicin damage.

The changes in the number of hair cells over time after the gentamicin damage is shown below.



	Week 1	Week 2	Week 4
Type 2 cells	16	59	33
Type 1 cells	0	0	15

The type 2 hair cells peaked at 2 weeks, then tailed off.
The type 1 hair cells only appeared between 2 and 4 weeks post gentamicin damage.

Supporting cell changes or nuclear activity identified about a regenerating hair cell at TEM and LM

The term ***'Mitotic Figure'*** is used as a general term to describe features seen at light microscopy and Transmission Electron Microscopy in a cell undergoing mitosis.

As previously mentioned the term covers features such as nuclear envelope dissolution and the presence of identifiable chromosomes and spindle fibres forming from the centrioles to chromosomes.

Other much less reliable changes which may indicate mitosis is about to occur are changes in the shape of a cell and in some cases changes in the position of the cell.

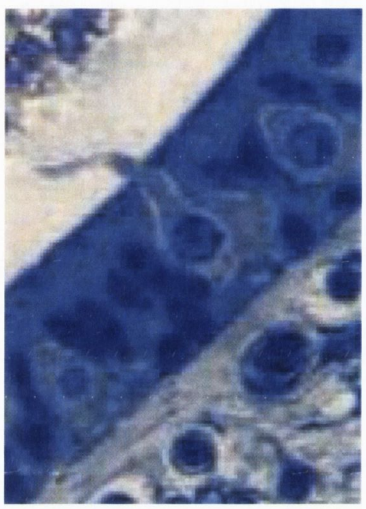
In no supporting cell seen at LM or TEM were definitive features of mitosis identified in this study.

Of the less reliable changes there was evidence of supporting cell nuclear migration towards the upper strata of the macula seen in all treated and in most untreated ears.

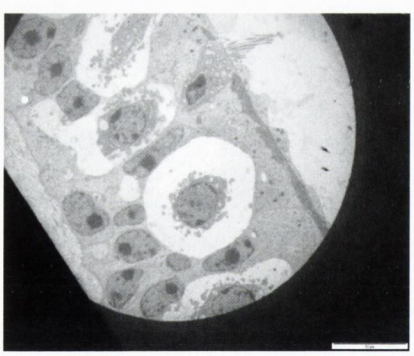
This was not dependant on the presence of a regenerating hair cell.

There was also a change of the shape of the supporting cells seen in almost all treated ears and most untreated ears.

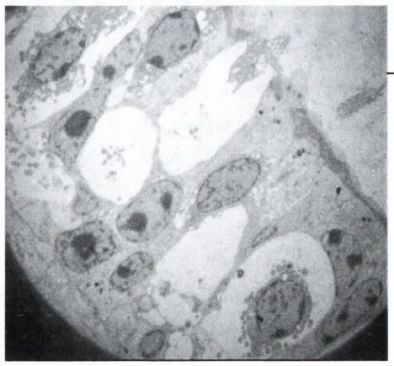
Lack of evidence of supporting cell Mitosis at TEM and LM



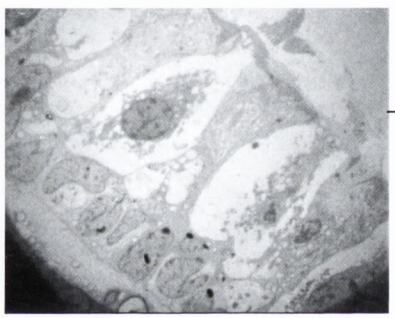
This regenerating hair cell has more supporting cells adjacent to it than is usually found or is necessary for metabolic stability (LM X 100).



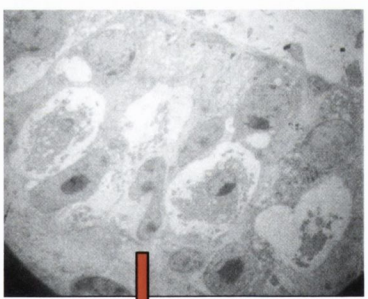
There appears to be a migration of supporting cell nuclei to the upper stratum of the macula (TEM X 1,000).



The migration of nuclei of the supporting cells was seen where there was a disruption of space around the cells caused by Hair cell apoptosis (TEM x 1,000)



This feature was far from consistent and just because there was space around supporting cells did not mean that there was supporting cell nuclear migration. (TEM x 1,500)



In other cases there was a suggestion of artifactual changes to explain the nuclear changes. Here the nucleus of a supporting cell but not the cytoplasm is distorted superiorly due to a space above the cell appearing when an adjacent hair cell underwent apoptosis (TEM X 1,000).



The red arrow indicates the area of magnification (TEM X 3,000).

Despite the apparent nuclear migration, there was no evidence of supporting cell mitosis at LM and TEM which would be necessary to support the premise that supporting cells nuclei migrate to the upper strata of the macula in order to undergo mitosis.

There was indirect evidence to support transdifferentiation of supporting cells to hair cells.

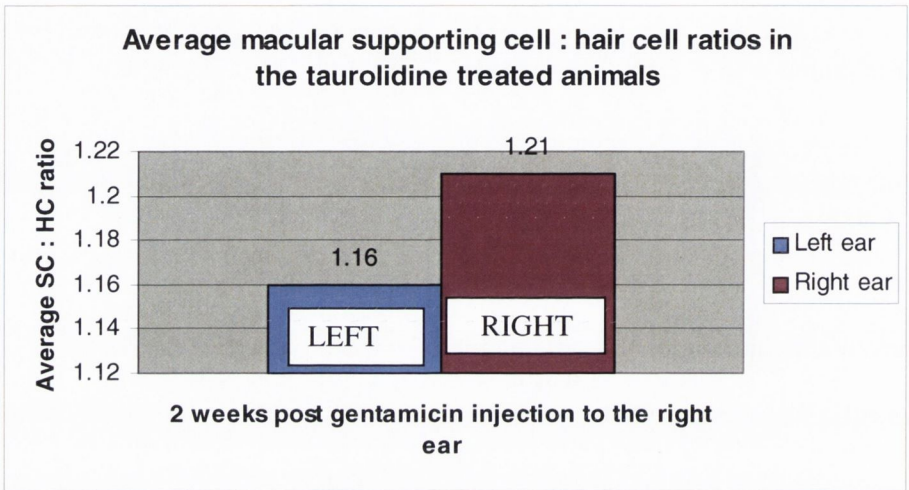
Taurine (Taurolidine) results on cell counts and mechanism of cell death

The 5 animals were sacrificed 2 weeks after the intratympanic gentamicin injection as at 2 weeks the maximal amount of damage to the ears can be expected (McConn-Walsh et al 2000).

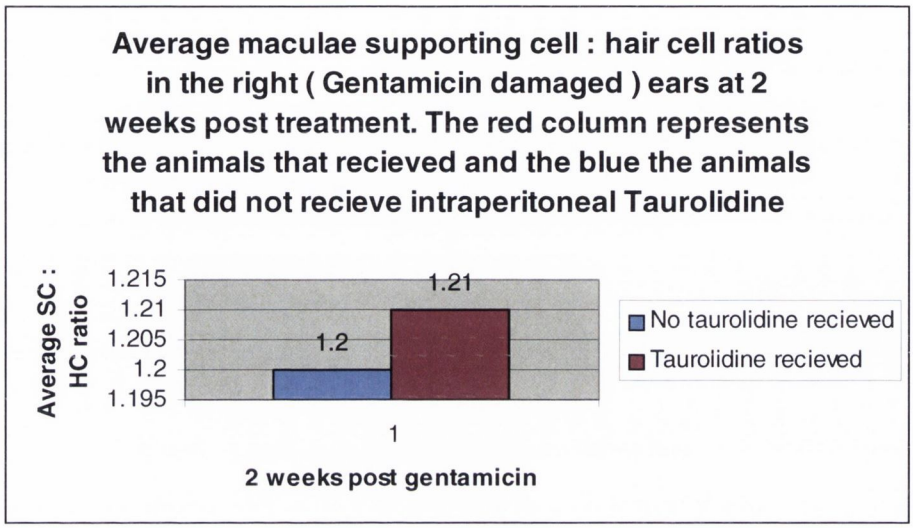
The results of TEM counts of supporting cells to hair cells ratios in the guinea pigs treated with intratympanic gentamicin to the right ears show that intraperitoneal tauroline is not protective to the ear.

It shows however that it appears at least morphologically to cause no increase in the cytotoxicity to the ear as suggested by previous studies.

The supporting cell : hair cell absolute counts were different from the cell counts in the bulk of this study however this is to be expected as the exact same areas of the maculae cannot be compared. The ratios however can be compared regardless of their site as a cross sectional representative section was taken in all cases.

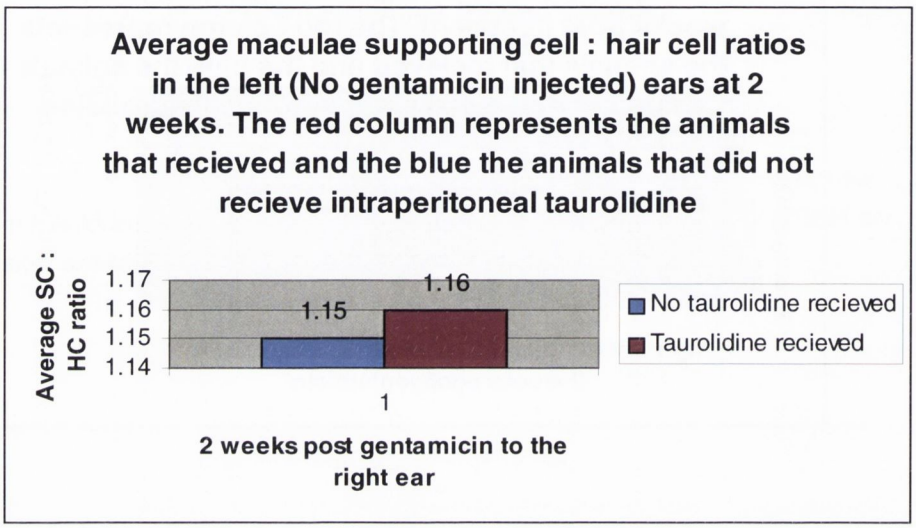


The figure above represents the graphic data of the taurolidine treated group (5 animals) harvested at 2 weeks.



The figure above represents the graphic data of both the taurolidine treated group (5 animals) and the 10 animals harvested at 2 weeks from the bulk data of this study. The mean ratio of supporting cells : hair cells in the right ears maculae (treated ears) of both groups are compared at 2 weeks post gentamicin damage.

Maximal ultrastructural damage is expected at 2 weeks in gentamicin damaged ears. Higher ratios indicate greater hair cell loss at 2 weeks.



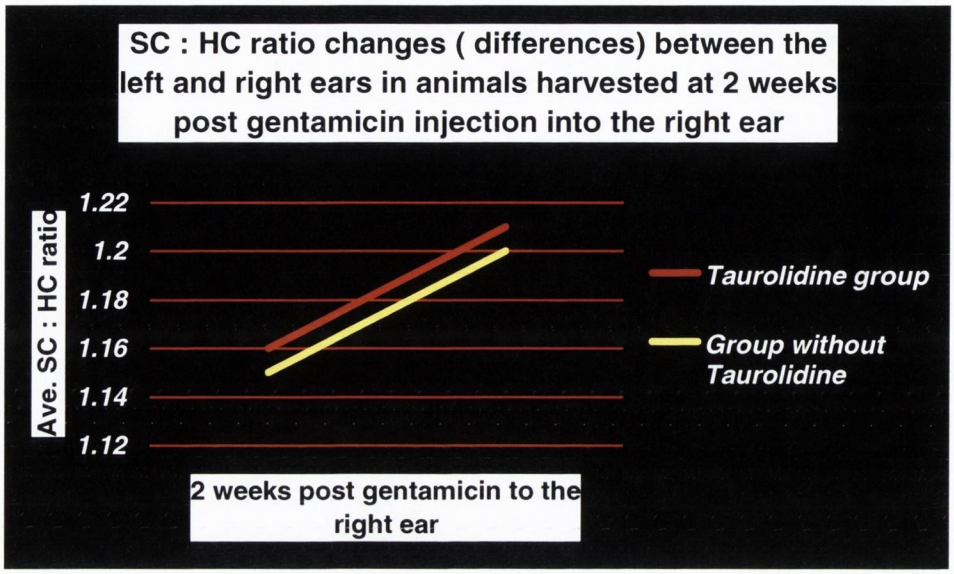
The figure above represents the graphic data of both the taurolidine treated group (5 animals) and the 10 animals harvested at 2 weeks from the bulk data of this study. The mean ratio of supporting cells : hair cells in the left ears maculae (untreated ears) of both groups are compared at 2 weeks post gentamicin damage.

The graph below shows the changes in the left sided (untreated ears) and right sided (treated ears) supporting cell : hair cell ratios in the group treated with taurolidine (red line) and with no taurolidine (yellow line).

No animal was sacrificed at the outset of the study and therefore no supporting cell : hair cell ratios in the normal untreated utricles counted. This ratio however should be 1:1.

It was hoped that the untreated left ears would be unaffected by the gentamicin and therefore an inbuilt control for reference for an changes from the normal expected ratio would be present.

Unfortunately some damage to the untreated maculae did occur through systemic absorption. Despite this a comparison between the gentamicin treated and non treated ears was still possible. The slopes of the graph in ascent is the key to interpreting the graph. Where the lines are parallel the relative ratios of supporting cells to hair cells are identical, where they diverge or converge significant differences are found.



Both graph plots have an equal rate of ascent (Slope).
The ratios changed in the Taurolidine group from 1.16 to 1.21 indicating a loss of hair cells when the left (untreated) and right (treated) were compared.
The same ratios of the non taurolidine group changed from 1.15 to 1.2 over the same period.
Therefore the taurolidine and non taurolidine **changes** between the left (untreated) and right (treated) utricles at 2 weeks are similar.

Thus there was no evidence based on cell counts that taurolidine was either cytoprotective or potentiated the potency of gentamicin on the treated ears at 2 weeks post gentamicin damage, the time when most hair cell loss is expected.

Technique of removing the utricle in an atraumatic fashion

This has been described on page 110.

Discussion

Method of Hair Cell death.

Hair cell loss in response to gentamicin induced toxic damage is known to occur in two ways - by apoptosis and extrusion.

In this study the TEM evidence refutes extrusion as even a minor but rather an incidental method of cell death. Apoptotic death is the fate of the majority of hair cells affected by the drug.

In both modes of hair cell loss, it appears that deletion of hair cells occurs without disruption of apical or basal tissue architecture or integrity. This may be important for subsequent repair and regeneration processes to operate (Li and Nevile 1995).

A third method of cell death - a combination of apoptosis and extrusion also occurs as is shown in this study.

We have confirmed that apoptosis is the major method of cell death.

Of particular interest in the field of hair cell death following initiation of the apoptotic pathway is the recent research on capsase inhibitors. Capsases are proteins specifically involved in the cell death pathway.

The fact that other recent research shows that hair cells exposed to gentamicin in the presence of caspase inhibitors appear to be preserved intact indicates that there may be a clinical role for these inhibitors in the future (Forge and Li 2000) (Van de Water TR, Lallemend F, Eshraghi AA et al 2004).

In the same way that capsase inhibitors protect cells from apoptotic death the future may reveal other agents which inhibit the apoptotic pathway.

This study suggests that by doing so they could in theory protect 99.99 % of all Hair Cells killed by gentamicin toxicity.

Taurine

Because the active metabolite of Taurolidine (Taurolin ®) is taurine, the terms taurolidine and taurine are used interchangably in the discussion. The exact function of taurine in the inner ear is unknown. It is however the most abundant amino acid in the perilymph of the inner ear and it is found in high concentrations in human electrogenic tissues such as the inner ear.

Taurine may be involved in the normal osmoregulation of the Organ of Corti and may function as a buffer when an osmotic insult is encountered.

It has also reduced apoptosis in damaged hepatocytes and has reduced reactive oxygen species which are known to induce necrosis. Because taurine has been shown to reduced reactive oxygen species and free radical oxygen scavengers have been shown to modulate the effects of aminoglycoside induced damage to the cochlea the hope was to see less damage in the form of supporting cell to hair cell ratios over time (a ratio of one to one being maintained over time indicating total protection).

The effect of taurine on the vestibular function of the inner ear has not been clinically investigated and the ultrastructural changes induced by aminoglycosides in the presence of supplementary taurine as evidenced by transmission electron microscopy has not to date been investigated either.

The mean ratio of supporting cells to hair cells changed in the Taurolidine group from 1.16 to 1.21 (the left side being the control ratio of 1.16. Ideally this should have been 1:1 however sytemic absorption meant that the left untreated side suffered some damage also). Taking the left side as the control the changes indicate a relative loss of hair cells in the right (gentamicin treated) utricle over the two week period. The supporting cells as in the rest of this study remained undamaged.

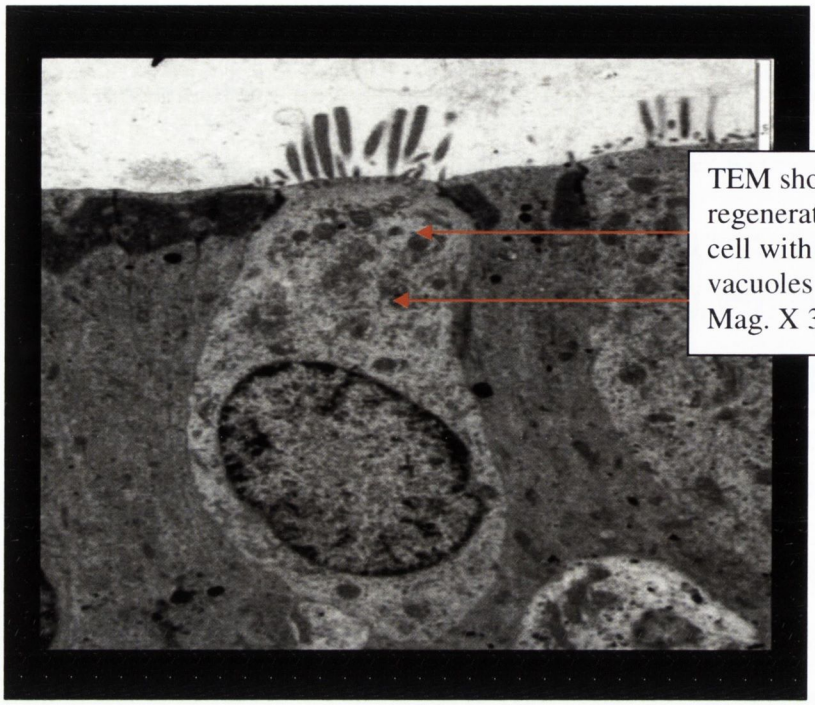
The supporting cell : hair cell ratios of the non taurolidine group ie. the group forming the major component of this study changed from 1.15 to 1.2 in the treated ears over the same period when the mean ratios for the left and right sides were examined. The supporting cells remained undamaged in this group also.

Therefore despite absorption of the gentamicin and subsequent unexpected damage to the untreated ears, when we regard the lesser damage sustained by the untreated ears as a baseline, the taurolidine and non taurolidine changes are similar with regard to the hair cell loss at 2 weeks post gentamicin treatment. As maximal hair cell damage is expected at this time (McConn Walsh et al 2000), this study suggests that there is no effect of taurine supplementation (via administration of taurolin) on the rate of hair cell death and ultrastructural changes in the guinea pig utricle induced by aminoglycoside ototoxicity.

Regeneration

Proposed methods of inner ear regeneration in the mammal include cell repair, supporting cell mitosis and differentiation and transdifferentiation of supporting cells from their original progeny to hair cells.

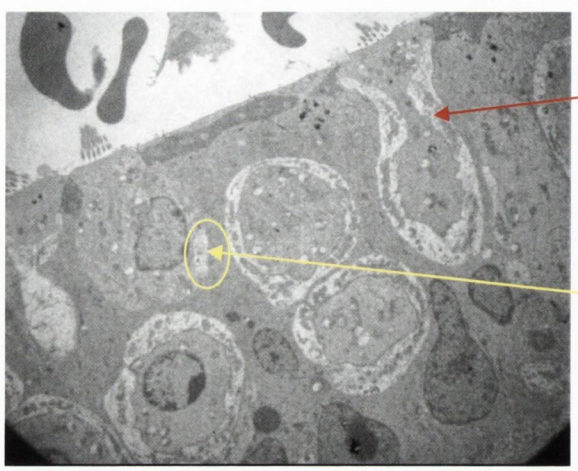
Using hair cell and supporting cell immunocytochemical markers in the Bullfrog, the fate of damaged hair cells and the origin of immature hair cells after gentamicin treatment in mitotically blocked cultures of the saccule has been examined.
Hair cells can undergo the early stages of apoptosis but not die after losing their stereocillia bundles. Sublethally damaged hair cells in this species remain in the epithelium for long periods, acquiring supporting cell-like morphology and immunoreactivity (Baurd et al 2000).
Ultrastructural analysis by TEM has shown that most immature hair cells in the Bullfrog sacules had autophagic vacuoles, implying that they originated from damaged hair cells rather than supporting cells (Baurd et al 2000).
Interestingly these autophagic vacuoles were present in 74 of the 108 regenerating type 2 hair cells hair cells (69 %) and in only 4 of the fifteen regenerating type 1 hair cells (27 %) in this study.



TEM showing a regenerating type 2 hair cell with autophagic vacuoles
Mag. X 3000

This study did not show *conclusive* evidence to support the theory of recovery of damaged hair cells in the mammalian inner ear, however in most type 2 regenerating cells there were autophagic vacuoles on TEM. The implications of this finding are discussed later.

Interestingly in almost all regenerating hair cells, the nerve terminals were intact.



TEM showing the nerve terminals in type 1 (red arrow) and type 2 (yellow arrow) are still intact.
Wallerian degredation has not yet occurred by the time regeneration has begun.
Mag x 2000

Supporting cell migration and mitosis

In both the treated ears and non treated ears, the ratio of supporting cells to hair cells was greatest at two weeks post treatment. In both groups the relative number of supporting cells was largest at 2 weeks post gentamicin damage only to decrease at 4 weeks post treatment.

It is known that at 4 weeks post gentamicin trauma to the inner ear the number of regenerating cells identified is greatest. Therefore it is likely that either the hair cells increased in number or the supporting cells decreased in number at 4 weeks post gentamicin or possibly both.

As previously stated the supporting cells were undamaged by the gentamicin induced toxicity as evidenced at TEM in this study. Therefore the only explanation for these findings is an increase in number of hair cells at 4 weeks.

Since no single mitotic cell was identified at Formvar ® TEM then the explanation for these findings is either hair cell repair or transdifferentiation of supporting hair cells occurs.

Interestingly many of the regenerating hair cell in mammals studies to date use Scanning Electron Microscopy (SEM) with regard to cell counts.

It would appear from this study however that many cells which at SEM may be regarded as possible regenerating cells or cells capable of repair are non viable when seen at TEM. Many cells cannot be regarded as regenerating cells due to the severity of damage identified.

This study showed some evidence to support the theory of cell repair as a method of inner ear hair cell regeneration in the mammal in the form of autophagic vacuoles.



Non viable hair cell with stereocillia that look viable. This cell is 4 weeks post damage by gentamicin. The cell may appear relatively healthy at SEM but is surely incapable of repair.

With regard to supporting cell nuclear migration in the macula the findings were highly variable.

Possible explanations for the finding of nuclei of supporting cells in the upper stratum of the macula include

1. The weakest evidence to support mitotic activity.

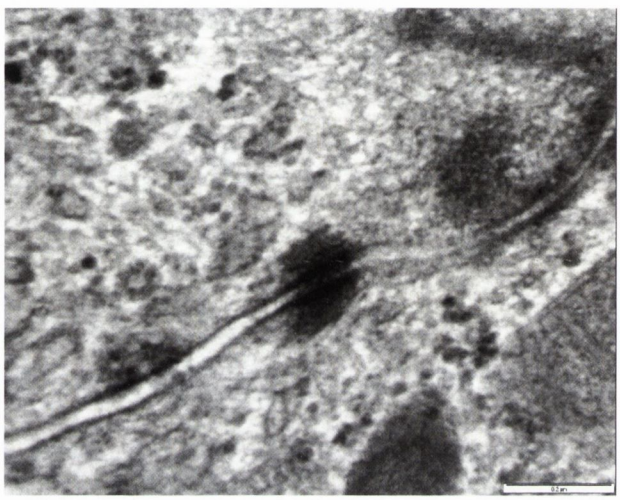
Therefore the suggestion that supporting cells were re entering the mitotic cycle is not supported in this study. The ability to identify proliferating supporting cells in the future studies may be enhanced by Key 67 stain shows cells dividing. In combination with Formvar TEM this stain could highlight exact positions on light microscopy specimens to search for ultrastructural changes at the same TEM examined specimen.

2. Another possible explanation is that the finding of nuclei of supporting cells in the upper stratum of the macula is that it is artifactual.

   Often the migration was seen about apoptotic irreparably damaged hair cells which had created space for the supporting cell nuclei to flow into. This would seem unlikely as in such cases it would be expected that the whole cell including its cytoplasm would bulge or migrate also. This was not the case.

There was no actual bulging of the whole supporting cells seen.

Even after chronic exposure to aminoglycosides for up to one month the tight junctions of the reticular lamina at the apex of the hair cells remains intact.



TEM showing intact tight junction at the reticular lamina. Mag x 10,000

In contrast the junctions between adjacent supporting cells have been shown to be severely damaged (McDowell et al.1989). This study confirmed this. This means that the apex of the supporting cells are anchored whereas the junctions between them are loosened. Under such conditions the contents of supporting cells may be found in areas such as is found in gentamicin damaged cells.

It has been shown that in invertebrates and birds hair cells are regenerated by the mitotic division of supporting cell progenitors and the differentiation of the resulting progeny into new hair cells and supporting cells (Baurd et al 2000).

Therefore it is likely that supporting cell transdifferentiation is a source of regenerating hair cells in birds. Further experimental findings in birds have suggested that direct transdifferentiation is a simpler, more rapid process that produces the first

new hair cells, and that mitotic regeneration is somewhat slower but ultimately produces most new hair cells (Robertson DW, Alosi JA, Contache DA. 2004). The identical morphology of regenerating hair cells from both pathways suggests that once hair cell fate is established, all new hair cells follow similar cellular processes during differentiation and reorganization into the regenerated epithelium.

It has been shown in Bullfrogs that transitional cells which express hair cell and supporting cell markers are seen near scar formations left behind when hair cells undergo gentamicin induced apoptosis. They seem to be created by some change in the supporting cells which are left behind when adjacent hair cells die.

Most of these cells have morphology and immunoreactivity similar to that of sublethally damaged hair cells (Baurd et al 2000). Indeed it is possibile that transitional forms of hair cell and supporting cell precursors may reside in the inner ear in a quiescent state until stimulated by damage (Morest DA, Contache D 2004).

Further evidence in support of transdifferentiation in amphibians was gathered when supporting cells from gentamicin damaged newt vestibules were prevented from entering mitosis and new hair cells were still generated. The results suggest that direct conversion of supporting cells into hair cells without an intervening mitotic event is a major mechanism of hair cell regeneration in the newt (Taylor RR, Forge A 2005).

A similar mechanism has been proposed for the hair cell recovery phenomenon observed in the vestibular organs of mammals.

This would mean that in mammals hair cells could regenerate from supporting cells without an intermediate mitotic phase with some supporting cells undergoing phenotypic conversion into hair cells without an intervening mitotic event.

This means that in gentamicin damaged inner ear maculae for example, supporting cell counts shortly after damage should significantly outnumber the hair cell counts

initially but over time and without evidence of mitosis the supporting cell counts should decrease and hair cell counts increase.

Such a situation would explain this study's experimental findings.

Type 1 and type 2 regenerating hair cells and the significance

Previous studies have suggested that regenerating hair cells have the morphology of type 2 hair cells (Forge et al 1993). From the cell counts in this study no type 1 regenerating hair cells were identified at 1 and 2 weeks post gentamicin ablation. All fifteen were identified in the samples examined at 4 weeks post gentamicin ablation

Of the 108 type 2 regenerating hair cells identified sixteen were seen at 1 week, fifty nine at 2 weeks and thirty three at 4 weeks post gentamicin ablation.

This data suggests that type 1 regenerating hair cells may regenerate later than type 2 cells however a more plausible explanation is that type 2 regenerating cells may somehow acquire the morphology of type 1 cells in time.

Type 2 hair cells are phylogenetically older than type 1 cells and thus the latter explanation for the findings would support a theory of regeneration.

This study's findings suggest that some of the type 2 regenerating hair cells which may arise from transdifferentiation of supporting cells may further differentiate into type 1 regenerating hair cells.

Conclusion

This study did not find evidence of actively dividing supporting cells to support the theory of supporting cell mitosis and differentiation as a source of hair cell regeneration in mammals.

No specific evidence of hair cell repair was found however the autophagic vacuoles found in regenerating cells may indicate an attempt at cell repair.

This study does however provide indirect evidence to support the method of transdifferentiation as the source of mammalian inner ear hair cell regeneration.

The evidence in support of direct supporting cell transdifferentiation as a means of hair cell regeneration in this study includes

1. The fact that these autophagic vacuoles are significantly more common in type regenerating hair cells (suggesting that by the time type 1 regenerating hair cells appear the vacuoles are beginning to disappear).
2. The supporting cell : hair cell ratio changes observed at 1, 2 and 4 weeks post gentamicin damage.
3. The lack of evidence of supporting cell mitosis.

The study' s conclusions are therefore

1. That the new Technique for atraumatic removal of the utricle is the best means of ensuring good samples for study by TEM. The new method of atraumatic utricle dissection will decrease the incidence of damage artifact in future studies of this nature as indeed it did in this study.
   Because it means that the utricle is never actually touched with a microforceps there is no possibility of crush damage to any specimen. Also as no specimen actually left the gluteraldehyde solution prior to TEM preparation, then the potential for drying artifact was eliminated. This technique should be utilised in all future studies of this nature.

2. That Formvar TEM is the best method for studying the cells both in terms of cell counts and ultrastructural examination in any future studies.

3. That this study shows non specific evidence to support hair cell repair as a method of regeneration (autophagic vacuoles in regenerating cells).

4. That the migration of supporting cell nuclei in response to gentamicin induced inner hair cell death appears to be nothing more than artifactual.

5. That regenerating type1 hair cells may develop from a more primative precursor which has the morphology of a type 2 hair cell.

6. That Taurolidine does not increase or decrease the TEM evidence for potentiating hair cell loss in gentamicin induced toxicity.

7. That direct transdifferentiation of supporting cells appears to be a potential origin to hair cell regeneration in mammals.

Future Studies

Future studies are already underway at the time of writing this thesis.

The five research fields which may lead to a breakthrough in inner ear hair cell regeneration in the mammal are growth factors, gene manipulation, heat shock protein, capsase inhibitors and stem cells.

Recent studies have shown the potential for geranylgeranylacetone (GGA) which can induce heat shock protein (HSP)70 in the vestibular organs of the guinea pig and reduce gentamicin ototoxicity. Administration of GGA may therefore protect vestibular sensory cells from gentamicin ototoxicity (Takumida M, Anniko M 2005). The induction of HSP70 by GGA may be a useful adjunct for the treatment of vestibular disorders.

The most exciting research into mammalian inner ear Hair Cell regeneration revolves around the possibility of stem cell induced recovery.

The vestibulocochlear nerve of adult rats and guinea pigs will probably support transplants of embryonic dorsal root ganglia and embryonic stem cells.

Even more exciting is the research around adult nerve stem cells. In addition to their innate potential to reform damaged neuronal tissue, the ability of nerve stem cell-derived cells to produce neurotrophins in the inner ear has also been demonstrated (Nakagawa T, Ito J. 2005).

It is not just nerve growth factors which show promise in manipulating implanted stem cells. It has been shown that differentiated adult mouse hair cells with a targeted deletion of Retinoblastoma 1 gene (Rb1) undergo mitosis, divide, and cycle to become highly differentiated and functional. Manipulation of the Rb pathway may ultimately lead to mammalian hair cell regeneration (Sage C. Huang M, et al. 2005)

Appendix

Stains and staining

Staining for electron microscopy

The specimen rests on its copper grid and this is floated specimen side down on a drop of 2% Uranyl acetate, a derivative of uranium for 20 minutes.

The specimen is then removed and washed with distilled water.

After thoroughly drying the specimen on clean filter paper specimen side up it is then floated on a solution of lead citrate for 10 minutes. These two agents are electron dense and act synergistically as heavy metals to stain the epon contained tissue but not the Epon. The specimen is then removed and rewashed with distilled water.

The specimen is then dried and stored for examination.

The copper grid specimens were dried on filter paper but the Formvar ® specimens were dried upright in blue tac. When dry all specimens were stored safely for later examination.



A 2% lead citrate solution is made by

1. Place 1.33 gms of lead nitrate (Pb(No3)2), 1.76 gms sodium citrate and 30 ml distilled water in a flask and ultrasonicate for 30 minutes.
2. Add 8 ml 1M sodium hydroxide (NaOH) and make up to 50 ml using distilled water.
3. Centrifuge for 15 minutes at 3,000 RPM.

A 2% urinyl acetate solution is made by

1. Add uranyl acetate powder 0.04 g to 2 ml 70 % ethanol.
2. Ultrasonicate until clear.
3. Centrifuge at 300 RPM for 15 mins
4. Pipette top 2 thirds of fluid to a clean container for future use.

Staining for light microscopy

The specimen is removed from the glass knife with a single small human hair mounted on a cocktail stick

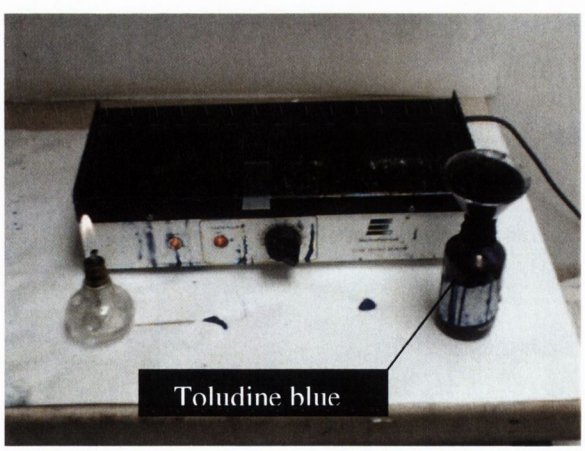
It is then placed on a drop of distilled water on a glass slide and heated on a heating plate to cause the specimen to unfold in the evaporating water.

The specimen was then annealed (stuck to the glass slide) by passing the slide over a naked flame to prevent it from being flushed away when washing the slide.

Toludine blue tissue stain is added and left on the slide until the rim of the toludine blue drop turned green (several seconds).

The specimen is then washed in distilled water and dried over the heater.

Finally the specimen is covered with microscopy glue and a glass cover slip for examination.



Making Toludine blue

1. Dissolve 10 % toludine blue in distilled water.

2. Add 1% Borax

3. Filter through filter paper before each use.

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