Chapter 1:

General Introduction

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GENERAL INTRODUCTION

Deafness is the most common of the sensory disorders with approximately one in every 850 children born suffering a permanent hearing impairment (Fortnum *et al.* 2001), and progressive deafness affecting 16% of the adult population (Davis *et al.* 1995; Steel and Kros 2001). Syndromes which include deafness as part of their collection of symptoms also contribute to the number of people affected, with over 430 different syndromes currently listed in the Online Mendelian Inheritance in Man (OMIM) database.

A number of aetiologies can contribute to hearing impairment, including environmental factors, exposure to ototoxins, genetic effects and developmental complications. However, it is estimated that half of all instances of deafness can be attributed to a genetic cause (Resendes *et al.* 2001; Morton 2002). Thus the study of the genes involved in the molecular mechanisms of hearing not only has the potential of elucidating the pathways and interactions which operate within the auditory system allowing us to hear, but also of identifying candidates for deafness disorders.

1.1 FUNCTIONAL ANATOMY OF THE EAR

1.1.1 The auditory system

The mammalian peripheral auditory system is composed of three distinct parts, the outer ear, the middle ear and the inner ear (see Figure 1.1).

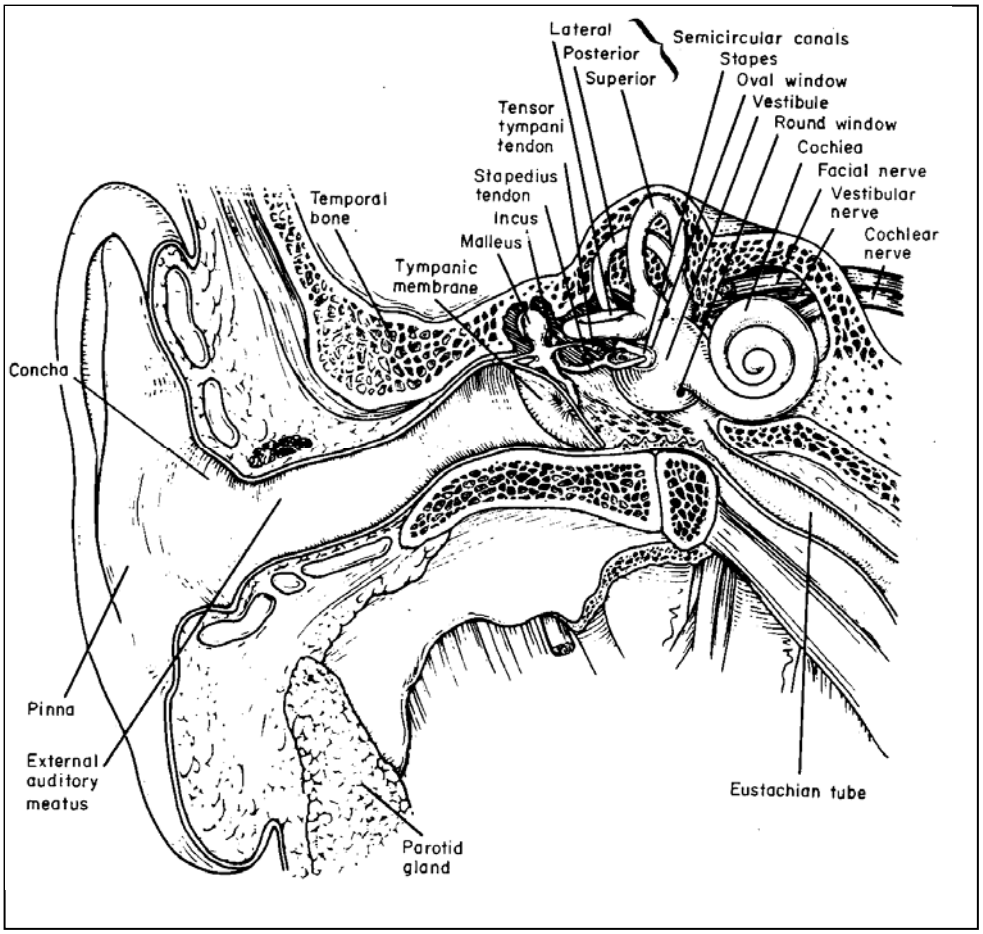

Figure 1.1: The structure of peripheral auditory system, comprising the external, middle and inner ears (from Pickles, 1988).

1.1.1.1 The external ear

The outer ear is formed by the fleshy auricle (in humans) or pinna (in animals) and the external auditory meatus (ear canal). The shape of the auricle allows airborne sound vibrations to be channelled into the ear canal, as well as enhancing high frequency sounds and aiding our ability to identify the directional origin of sound (Pickles 1988). The ear canal then conveys vibrations towards the tympanic membrane where they are transferred to the middle ear.

1.1.1.2 The middle ear

The middle ear is an air-filled recess within the temporal bone. Within this cavity are located the three auditory ossicles, the malleus, incus and stapes, as well as the tensor tympani and stapedius – the muscles which hold them in place. The remainder of the middle ear is made up by the Eustachian tube and the tympanic membrane.

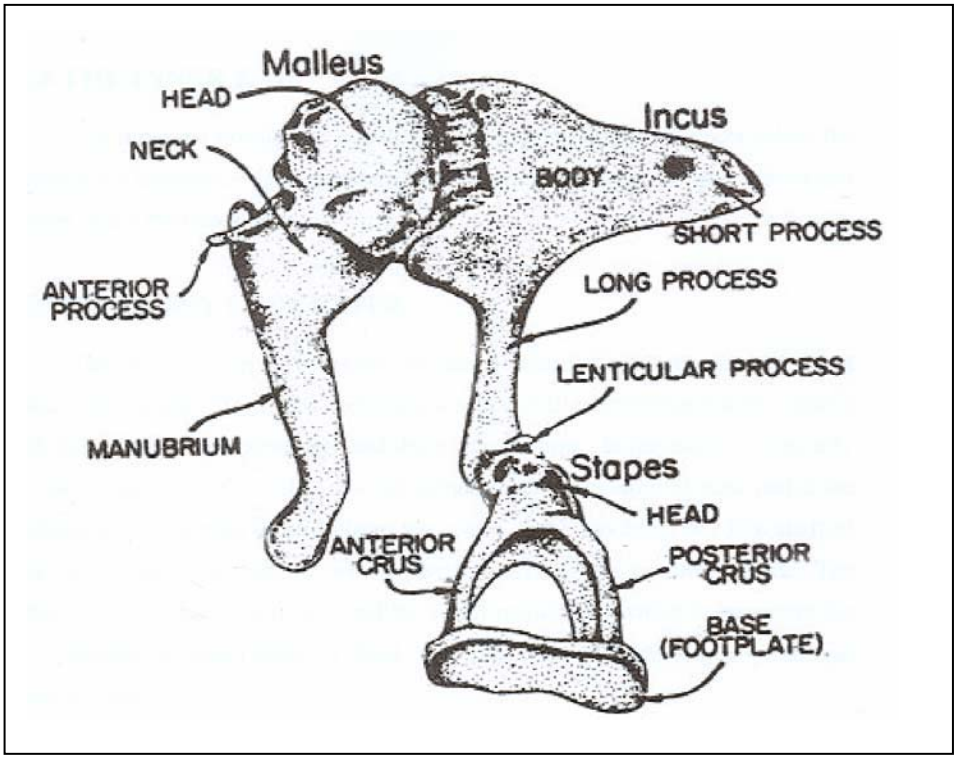

Figure 1.2: Schematic of the human ossicular chain of the middle ear showing the anatomical landmarks of each bone. The ossicles form a continuous chain spanning the middle ear cavity with the manubrium of the malleus contacting the tympanic membrane and the footplate of the incus inserting into the oval window of the cochlea (from Schuknecht 1974).

The tympanic membrane is a tri-laminar structure, consisting of an outer layer of ectoderm, an internal lining of mesoderm and an inner layer of endoderm. Its external surface is in contact with the outside environment via the external auditory meatus, whilst its inner surface connects with the manubrium of the malleus (Figure 1.2). The head of the malleus articulates with the body of the incus, and the lenticular process of the incus connects with the head of the stapes. The ossicular chain spanning from the outer ear to the inner ear is completed by the insertion of the footplate of the stapes into the oval window of the cochlea. Thus vibrations carried through the external auditory meatus to the tympanic membrane are transmitted to the inner ear. In addition, the lever action of the ossicular chain, combined with the transfer of vibrations from the large, low impedance tympanic membrane to the smaller, higher impedance oval window results in an amplification of the sound as it reaches the cochlea (Pickles 1988).

1.1.1.3 The inner ear

The inner ear is made up of two labyrinths – the bony labyrinth and the membranous labyrinth, both of which are encased by a protective bony shell called the otic capsule. The bony labyrinth can be divided into 3 distinct areas: the vestibule; the three semi-circular canals and the cochlea (Figure 1.3a). Within these lie the separate areas of the membranous labyrinth: the saccule; the utricle; the semicircular ducts and the cochlear duct or scala media (Figure 1.3b). Functionally, the inner ear can be divided into two systems. The vestibular system which is concerned with the mechanisms of balance comprises the semicircular canals, the vestibule and the membranous sacs and ducts they enclose. The snail-like cochlea forms part of the auditory system, with the oval window of the bony labyrinth situated at its base to allowing transmission of sound from the outer and middle ears. The development of the inner ear is discussed in Section 1.2.3.

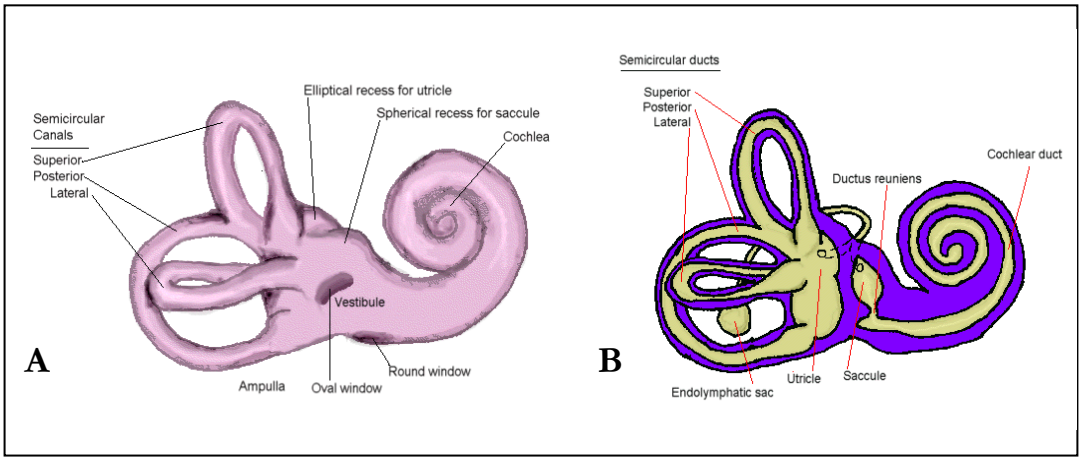

Figure 1.3: The gross structure of the inner ear, showing the bony labyrinth (A) and the membranous labyrinth (B)
From http://medic.med.uth.tmc.edu/Lecture/Main/ear.htm

1.1.2 The cochlea

The cochlear duct is a spiral-shaped bony tube surrounding a central core called the modiolus. In humans the spiral is two and three quarter turns in length, in the mouse it is one and three quarters. The cochlear duct is divided along its length into three chambers, the scala vestibuli, the scala tympani and the scala media (Figure 1.4). The scala vestibuli and scala tympani form the outer layers of the duct and both contain perilymph which is high in sodium ion (Na^+) concentration and low in potassium ion (K^+) concentration like most other extracellular fluids. Between them lies the scala media, a cavity which is triangular in cross section and filled with endolymph high in K^+ concentration and low in Na^+ concentration, similar to the intracellular fluids of neurons.

The division between the scala media and the scala tympani below it is formed by the basilar membrane. This structure is composed of connective tissue, with a distinct basement membrane separating it from the overlying organ of Corti. Above the scala media the boundary is formed by Reissner's membrane, while the external wall attaches to the wall of the bony labyrinth and is termed the lateral wall. This wall includes the spiral ligament and stria vascularis which play important roles in recycling endolymph and maintaining the ion gradients within the scala media.

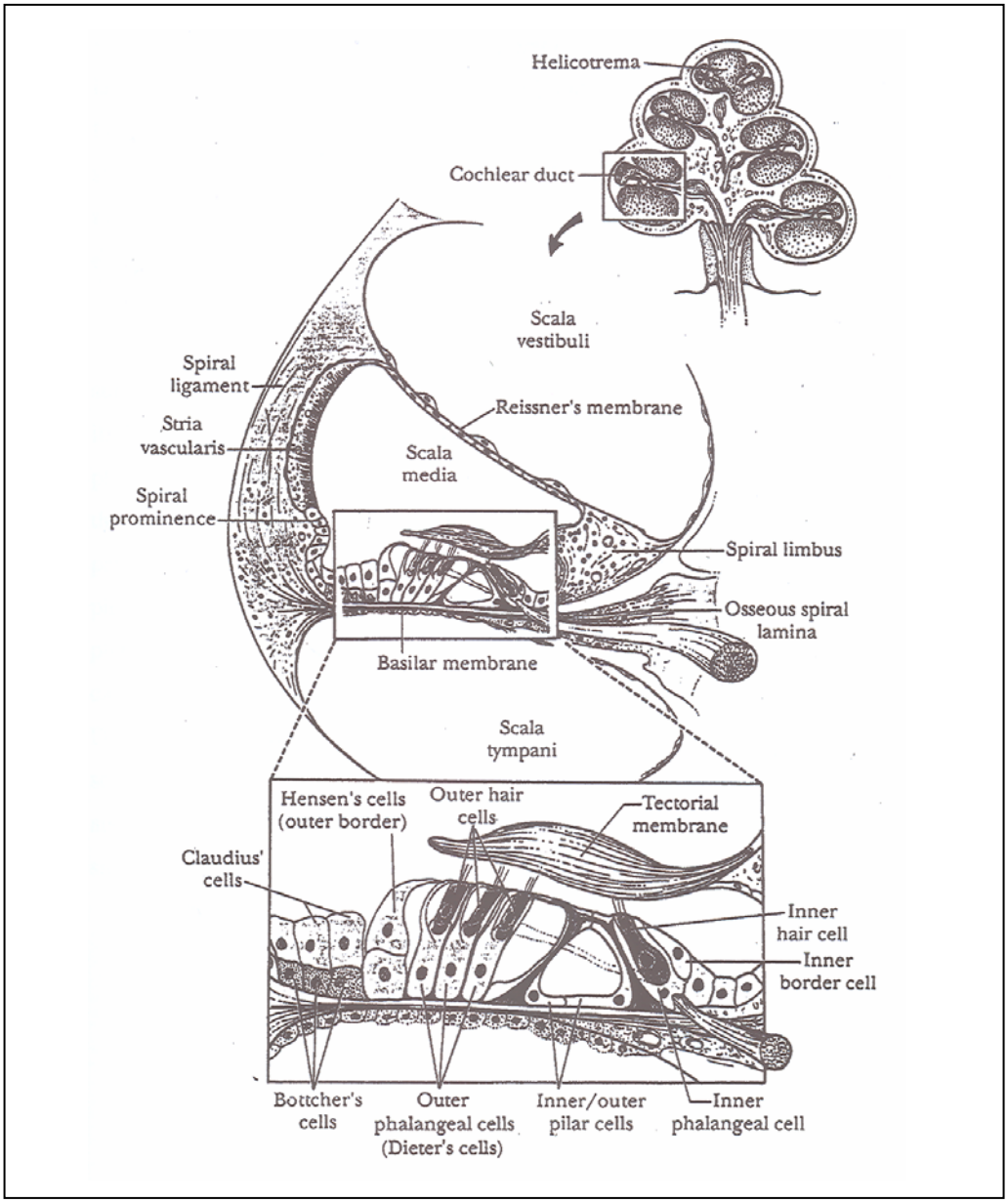

Figure 1.4: Diagrammatic representation of a cross section through the cochlear duct emphasising the location of the organ of Corti (from Goodhill 1979).

1.1.2.1 The organ of Corti

The organ of Corti is the highly specialised sensory patch of the cochlea and is composed of sensory hair cells and supporting cells. It rests on the basilar membrane and extends through the length of the cochlea within the scala media (Figure 1.4). Hair cells can be distinguished by the bundles of stereocilia on their apical surfaces and form two distinct types; the inner hair cells (IHCs) and outer hair cells (OHCs). The IHCs are arranged in a single row towards the modiolar edge of the organ of Corti, while the OHCs are ordered in three parallel rows (sometimes up to four rows in the apex) slightly further away from the central core of the cochlea (Figure 1.5). IHCs have a flask-shaped cellular body and a centrally located nucleus and account for 90-95% of the afferent innervation leading to the cochlear nucleus (Spoendlin 1972). The OHCs are longer and cylindrical in shape, and have an abundant efferent nerve supply. However, the most noticeable difference between the two types of hair cells is the arrangement of the stereocilia on their surface. The IHCs have their stereocilia arranged in a slightly crescent manner, whilst the stereocilia on OHCs are arranged in a distinctive "v" shape in the apex, widening to form a "w" shape in the base. In general the stereocilia of IHCs are longer and of larger diameter than those of the OHCs, which tend to be short and narrow. The stereocilia of both hair cell types form a "staircase" gradient with the tallest stereocilia at the outer edge of the cell. Stereocilia are composed of tightly packed actin filaments, making them relatively strong and rigid. Between the rows of stereocilia exist tip links and numerous lateral links which preserve the integrity of the bundle and also mean that the stereocilia in a bundle all move together. The deflection of the stereocilia bundle towards the tallest stereocilia row produces an excitatory response, as discussed later.

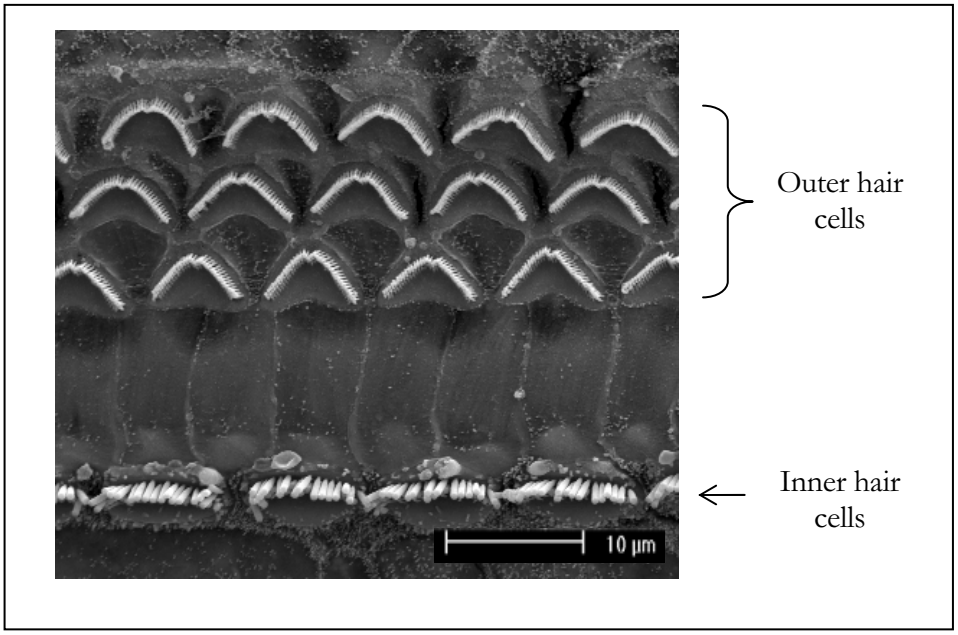

Figure 1.5: Scanning electron micrograph showing the apical surface of an adult mouse organ of Corti. Outer hair cells are arranged in three parallel rows and possess thin-shafted stereocilia which are arranged in a characteristic "V" or "W" formation. Inner hair cells form a single row and have thicker stereocilia organised into a slight crescent shape.

The sensory hair cells in the organ of Corti are surrounded by a variety of different supporting cells. Lying between the single row of IHCs and the first row of OHCs are two rows of microtubule-packed inner and outer pillar cells. As well as separating the two hair cell types, these cells give the organ of Corti rigidity along its length. The upper ends of the pillar cells form a plate called the reticular lamina which forms the division between the endolymph of the scala media and the perilymph of the scala tympani below. Surrounding the pillar cells are phalangeal cells. Inner phalangeal cells surround the inner IHCs, while outer phalangeal cells (also called Deiter's cells) support the OHCs, forming a cup at their base and extending fine microtubule-filled processes towards the reticular lamina which serve to separate the three rows of OHCs. Hensen's cells form the outer border external to the OHC rows, while inner border cells are situated between the row of IHCs and the modiolus.

The tallest stereocilia of the OHCs are embedded in the fibrous and gelatinous tectorial membrane. This structure is anchored to the spiral limbus

and forms a flap which covers the organ of Corti. The IHCs are not embedded in this matrix, but instead fit loosely into a groove on its under surface (Pickles 1988).

1.1.2.2 Auditory transduction

Auditory transduction refers to the transformation of the tiny vibrational movements of the basilar membrane and the organ of Corti which rests upon it into electrical impulses. The sound waves that enter the cochlea via the outer and middle ear at the oval window cause the displacement of endolymph in the scala media which is accommodated by the round window. This movement of the fluid surrounding the basilar membrane causes it to be displaced, with the frequency of the sound determining the pattern of movement. Sounds of a low frequency result in a vibration pattern which peaks in the apex of the cochlea, while high frequency tones produce more vibration in the basal region. This arrangement is described as tonotopic organisation.

The movement of the basilar membrane in turn causes displacement of the stereocilia bundles on the surfaces of the sensory hair cells. This displacement occurs because the tectorial membrane in which their tips are embedded has a different pivot point from the basilar membrane which forms their base. Thus when the membranes are displaced, the stereocilia also undergo displacement.

Mechanical stimuli applied to a hair bundle elicit electrical responses by causing transduction channels to open or close. When the stereocilia bundle is deflected towards the tallest row of stereocilia, tension in the tip-links which connect adjacent stereocilia exerts force on the transducer channels and causes more of them to open, thus allowing an influx of extracellular K^+ and Ca^+ which causes depolarisation of the cell. A stimulus which brings about a movement of the stereocilia towards the short edge of the bundle has the opposite effect, causing the transducer channels to close and hyperpolarising

the cell. The degree of polarisation in turn affects the rate of release of a synaptic transmitter which causes the transmission of a pattern of action potentials to the brain. These are carried along an afferent nerve fibre which contacts the base of the cell, and encodes information regarding the intensity, time course and frequency of the sound stimulation.

1.1.3 The vestibular system

The vestibular system is concerned with the perception of static and dynamic equilibrium which is necessary for balance and spatial orientation. Static equilibrium relates to the position of the body in relation to gravity, while dynamic equilibrium is concerned with the maintenance of body position in response to movement. It comprises the semicircular canals and the otolithic organs, the saccule and the utricle, as well as the endolymphatic sac and duct (Figure 1.3). The endlymphatic duct joins the endolymphatic sac to the utricle and plays a role in fluid homeostasis; the anatomy and function of the remaining components are described below.

1.1.3.1 The semicircular canals

Within the bony semicircular canals lie the lateral, anterior and posterior semicircular ducts (Figure 1.3). Both ends of each canal join to the vestibule, while the rostral end opens out into an ampulla, an expanded cavity containing a sensory patch known as the crista ampullaris. Each of these is an elongated epithelial structure situated on a ridge of supporting tissue arising from the wall of the ampulla, with its surface populated by three distinct cell types. The first of these are supporting cells, characterised by a tall columnar shape and short microvilli on the apical surface. The other two cell types are sensory hair cells, which are classified as type I or type II depending on their morphology and innervation. Type I cells are flask shaped, with an afferent nerve chalice and some efferent nerve fibres surrounding the baso-lateral

region. Type II cells are cylindrical in shape, and possess both efferent and afferent innervation at their base.

Both type I and type II cells possess stereocilia on their apical surface. These are stiff actin-filled microvilli which are organised into a "staircase" shape, with taller projections at the back, gradually decreasing in height towards the front. The stereocilia of the hair cells project into a cone-shaped gelatinous membrane called the cupula. Each cell also has a single kinocilium – a cilium containing the classic 9+2 arrangement of microtubules at its core – which is always located on the utricular side of the hair cell.

The semicircular ducts and their associated cristae are responsible for the detection of changes in the direction and rate of movement of the head, known as rotational acceleration and deceleration. In order to account for all planes of movement, the superior and posterior canals are in vertical planes perpendicular to each other, while the lateral canal is in a near-horizontal plane. Each crista is located perpendicular to the plane of the duct with which it is associated. Rotation of the head in the plane of that semicircular canal causes movement of the endolymph within it, which in turn displaces the cupula. This causes movement of the embedded stereocilia bundles towards or away from the kinocilium, resulting in depolarising or hyperpolarising receptor potentials respectively and the release of a neurotransmitter at varying rates, initialising neural impulses towards the central vestibular system.

1.1.3.2 The otolithic organs

The utricle and saccule are dilated regions of the central vestibule, each containing a sensory patch termed a macula. The two maculae are located perpendicular to each other and are composed of supporting cells and type I and type II sensory hair cells similar to those found in the cristae. The stereocilia of the hair cells are embedded in a gelatinous layer of glycoprotein called the otolithic membrane, a sheet-like structure which is thought to be

secreted by the supporting cells. The upper surface of this membrane contains minute crystalline bodies composed of calcium carbonate and protein which are known as otoconia.

The maculae are responsible for the maintenance of static equilibrium – the detection of gravity – which is of particular importance when other cues as to spatial orientation are lacking, such as when the eyes are closed, or in the dark or under water. Movement of the head causes the otolithic membrane, weighed down by the otoliths, to move in relation to the hair cells beneath them, giving rise to displacement of the stereocilia. As in the cristae and the cochlea, this causes increased excitation when the movement is towards the kinocilium and reduced excitation when the movement is away from the kinocilium.

1.2 DEVELOPMENT OF THE EAR

1.2.1 Development of the external ear

The pinna of the external ear arises from the ectoderm of the first and second pharangeal arches. During the fifth week of human development three pairs of auricular hillocks arise from these tissues. These then proceed to enlarge, differentiate and fuse to give the final shape of the pinna. The external auditory meatus is derived from a deepening of the first pharangeal cleft during the sixth week. This is followed by cell proliferation and canalization of the deepening tube.

1.2.2 Development of the middle ear

Beginning at the seventh week of gestation (E13.5 in mice), the ossicles develop from neural crest derived cells from the first and second arches which condense to form cartilage before being converted to bone by the process of endochondral ossification. During the ninth month of development pharangeal endoderm expands to enclose the ossicles, forming the tympanic cavity, with the connection to the pharynx being maintained by the Eustachian tubes. Meanwhile, the pharangeal membrane separating the tympanic cavity from the external auditory meatus develops into the tympanic membrane, a structure composed of an outer layer of ectoderm, an internal lining of mesoderm and an inner layer of endoderm. Once the ossicular chain has been established, the ventral end of the malleus becomes attached to the eardrum and the footplate of the stapes becomes attached to the oval window of the bony labyrinth, thus forming a continuous connection between the outer and inner ears.

1.2.3 Development of the inner ear

In the mouse the first sign of inner ear development is seen between embryonic day E8 and E8.5, corresponding to four weeks of development in

the human embryo. This takes the form of a thickening in the ectoderm adjacent to the rhombomeres 5 and 6 of the embryonic hindbrain which is termed the otic placode (Figure 1.6C). The derivation of this structure has been the subject of much and varied research. It is thought to be of ectodermal origin, although in the absence of surrounding tissues ectoderm has been found to be unable to develop an otic placode (Jacobson 1963). Hence the role of the surrounding tissues in inducing its formation has been considered, the main candidates for this role being the mesoderm, neural plate, endoderm and notochord. The main mode of study employed was that of transplantation and in this way the tissues necessary for the specification of the ectoderm have been characterised.

The most likely ectoderm to form ear vesicles was that taken from tissue between mid and late gastrulation and from near the site where they would be expected to form, thus implying that there exists a specified otic field before the otic placode becomes visible. Jacobson (1963) also suggested that this could be pre-empted by a broader placodal field, including the tissue which will later differentiate to become ear, nose and lens placode.

It was found that several of the surrounding tissues were capable of inducing an otocyst when transplanted individually with ectoderm. However, the most successful induction occurred when several tissues were present, indicating a more complicated mechanism of induction than was previously assumed.

Following the establishment of the otic placode, the structure begins to invaginate, forming an otic cup or pit (Figure 1.6D). This pit continues to deepen until it is completely closed off and an otic vesicle or otocyst is formed (Figure 1.6E). During this time the cochlear and vestibular neurons are formed by delamination from the otic cup, initially existing as a combined cochleo-vestibular ganglion but later splitting to form separate ganglia. There follows a period of much proliferation and the structure becomes enlarged, soon after which there begins the process of differentiation when

morphogenesis and patterned development of specific cell types will take place (Figure 1.6F).

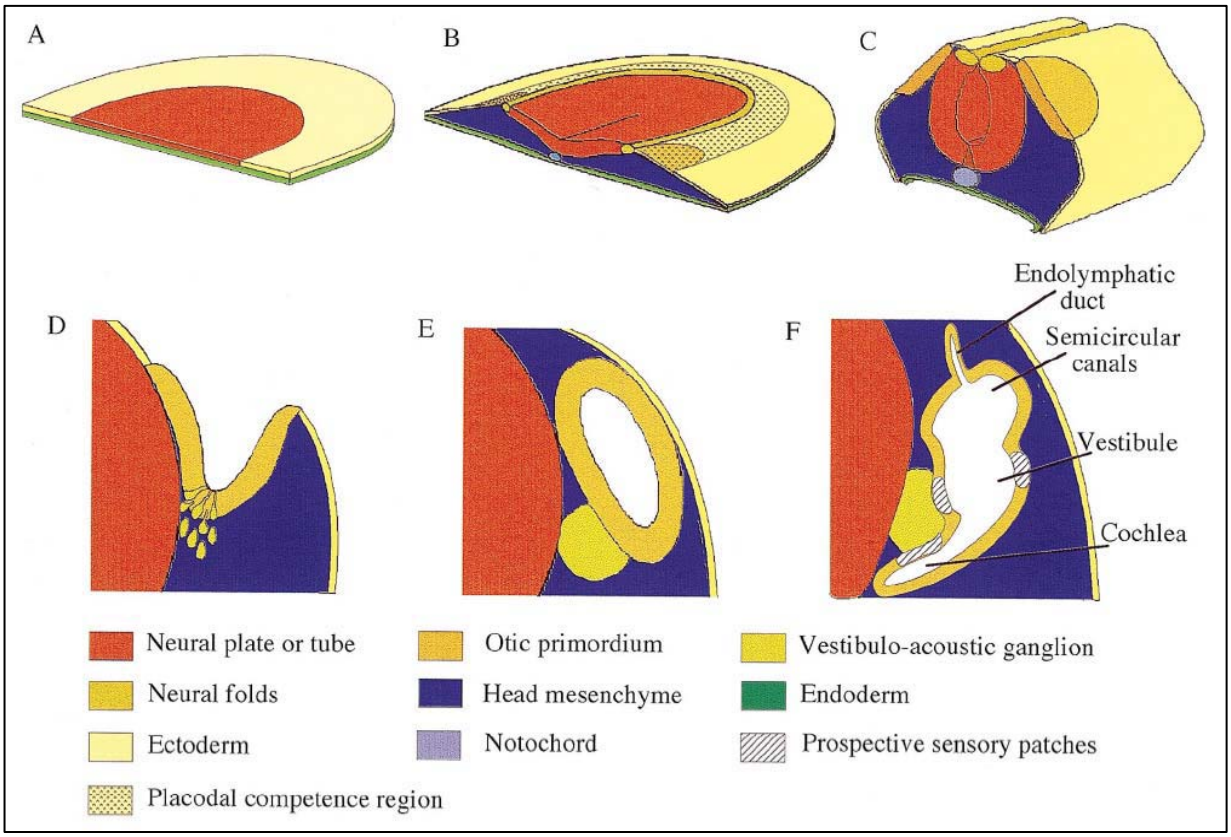

Figure 1.6: Summary of inner ear early development (from Torres and Giraldez 1998)

A) Pregastrula, the prospective neural plate is indicated in red.
B) Head fold, the prospective otic placode is indicated in orange and the prospective multiplacodal ectoderm stripe is stippled.
C) Otic placode.
D) Otic cup, neuroblast appears differentiating from the otic epithelium.
E) Otic vesicle.
F) Otocyst differentiation, the primordia of the different anatomical regions are as indicated.

1.2.3.1 Development of the organ of Corti

The organ of Corti forms a part of the cochlear duct which coils around inside the cochlea. At embryonic day E11.5 in the mouse, the cochlear duct begins to form from a tubular pouch which extends from the otocyst. The cochlear duct develops thickened epithelial areas, the greater and lesser ridges, from which the organ of Corti develops. It is thought that the greater epithelial ridge will give rise to the inner hair cells (IHCs), and the lesser epithelial ridge to the outer hair cells (OHCs).

The base of the organ of Corti is first to develop, and the tube continues to coil around so that by E13.5 in the mouse 1.75 turns have been made, but no cellular differentiation can yet be observed. Between E14.5 and E18.5 the tectorial membrane begins to form and the epithelial cells begin to specialise and mature so that eventually the IHCs and OHC s can be distinguished from the supporting cells by their stereocilia which first become apparent at E17.5.

When the mouse is born following approximately 20 days of gestation the organ of Corti is not yet fully formed. At birth the tunnel of Corti and Nuel spaces – the fluid-filled spaces between the pillar cells and surrounding the OHC cell bodies respectively – are absent. At postnatal day 6 the tunnel of Corti begins to open at the base as pillar cells devlop, slowly progressing up to the apex so that it is fully open by P10. Nuel spaces also develop during this time.

The IHCs and OHCs are themselves immature at birth, and undergo further maturation up to P14. Microvilli shorten or are resorbed, kinocilia disappear, synapses with nerve endings develop and the inner pillar cells expand between the rows of IHCs and OHCs to give them their distinctive mature appearance.

1.2.3.2 Development of hair cell stereocilia

The stereocilia first develop in the mouse at E16.5 as short, thick microvilli covering the surface of the hair cells with over 200 per cell. This is far more than would be expected in a mature hair cell and indeed many of these subsequently regress. The stereocilia vary in height, with the tallest being near the kinocilium and the rest forming graduations down towards the cell surface. Tip links and side links between the stereocilia are thought to develop as this staircase pattern evolves, with the remaining unlinked stereocilia being reabsorbed by the cell. Mature-looking stereocilia are visible at P14, when the kinocilium has also been reabsorbed. By P20, the stereocilia are fully mature.

1.2.3.3 Development of the semicircular canals

The semicircular canals initially develop from bilayered outpocketings of epithelium from the otic vesicle at embryonic day E11.5 in the mouse. The superior and posterior semicircular canals will develop from a single dorsally directed outpocketing, while the horizontal canal develops later and from a separate laterally directed outpocketing. The two layers of epithelium in the central regions of these pockets, termed canal plates, join each other and fuse. This movement is thought to be brought about by the production of glycosaminoglycan by the canal plate epithelial cells which is secreted onto the underlying mesenchyme (Haddon and Lewis 1991). During this fusion the epithelium appears to vanish, leaving a hollow rim of epithelium surrounding the periphery of the original outpocketing (Figure 1.7). Martin and Swanson (1993) showed that the disappearance of these cells is due to their retraction into the canal epithelium adjacent to the fusion site in the mouse, whereas in chick it is thought to be due to programmed cell death. The crucial developmental stages of the semicircular canals are over by embryonic day E13.5, by which time the inner ear labyrinth is fully formed in miniature.

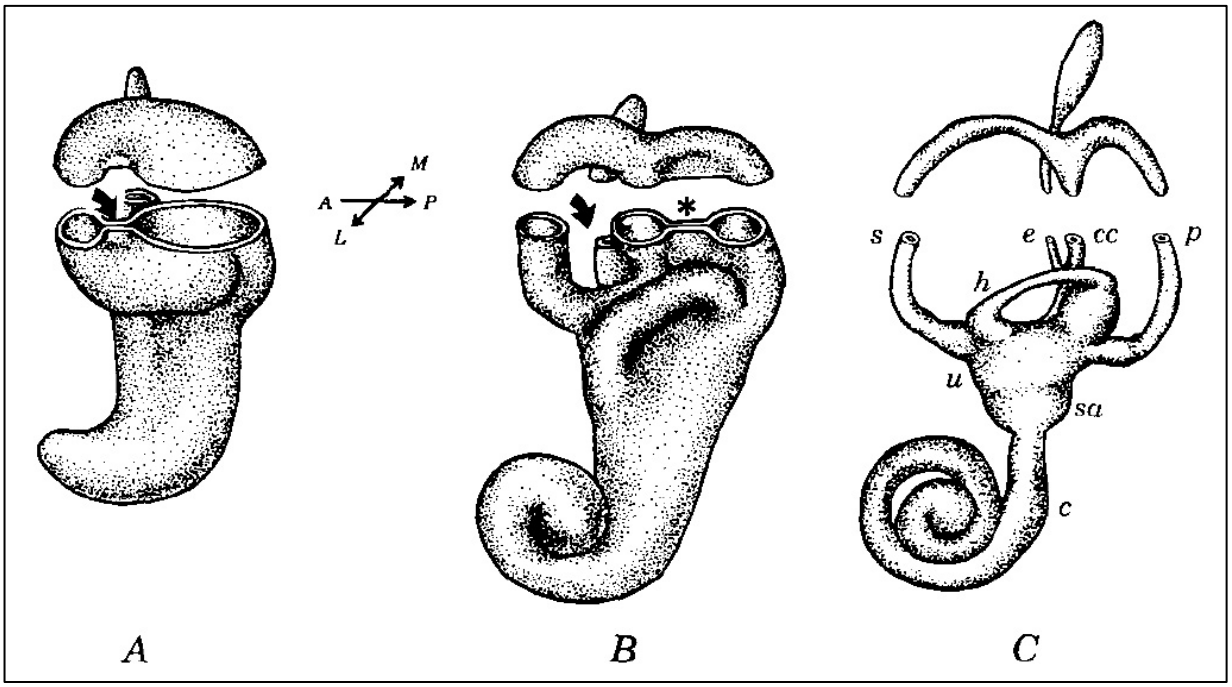

Figure 1.7: Inner ear morphogenesis during semicircular canal development (Martin and Swanson 1993)
A - The walls of the canal plate have met in the region of the superior semicircular canal (arrow).
B - The fused layer of canal plate epithelium has disappeared, posterior canal walls have come together and are opposed.
C - The superior and posterior semicircular canals are no longer fused but are united by the shared crus commune.

Key:
s - superior semicircular canal
p - posterior semicircular canal
h - horizontal semicircular canal
cc - crus commune
e - endolymphatic duct
u - utricle
sa - saccule
c - cochlea

M - medial
L - lateral
A - anterior
P - posterior

1.2.3.4 Development of the maculae

At embryonic day E13.5 the maculae can be distinguished from the remaining epithelium of the membranous labyrinth by the microvilli on their surface. Each hair cell also possesses a single kinocilium which is distinct from the microvilli in its exaggerated length and thickness. By E14.5 the hair cells have developed ciliary tufts and the kinocilium has become polarised slightly off centre on the hair cell surface. The maculae then begin to grow in surface area, a change thought to be due to the migration of the supporting cells from a multi-layered structure to a monolayer. Alongside this change the number of ciliary tufts increases so that by E16.5 it is possible to see well-developed tufts in the central striolar zone and more immature tufts at the periphery. The otoconia also appear at this stage, and continue to increase in number until birth. From this stage onwards the maculae continue to increase in size, while the central-peripheral gradient of development persists.

1.2.3.5 Development of the cupulae and cristae ampullares

At around E13.5 in the embryonic stage of mouse development the epithelial ridge, which will later be the site of the crista ampularis, becomes identifiable. At this time the ampullae may also be distinguished as a small dimple on the surface of the epithelium. Between E14.5 and E16.5 the gelatinous membranes which form the cupulae begin to develop via the secretion of material from the surface cells until by E16.5 they span the space between the cristae and the roofs of the ampullae.

Meanwhile the ampullae acquire a coating of microvilli, with the central region of the cristae also exhibiting polarised ciliary tufts. Kinocilia are also present. At E15.5 the size of the cristae and the number of hair bundles have both increased. In the cristae of the superior and posterior canals the torus can be seen beginning to form in the shape of a bulge separating the cristae into two parts. The crista itself is also altering in morphology, becoming

cylindrical from its previous flat appearance, and the ciliary tufts continue to spread and to enlarge. There are fewer tufts at the base than at the apex of the crista, and at birth this pattern is still seen, but with additional new immature tufts at the top of the cristae.

1.2.3.6 Genetics of hair cell development

The hair cells in the organ of Corti, the maculae and the cristae all develop from an initial sensory primordium which becomes distinct from the surrounding cells (reviewed by Fekete 1996). From this sensory primordium will arise both the auditory hair cells (AHCs) and the various types of supporting cells (SCs) which together make up the sensory epithelia. The fate of these cells is thought to be specified by lateral inhibition mediated by Delta-Notch signalling. Evidence suggests that the *Delta/Serrate/Lag2* (DSL) family ligands *Delta* and *Jagged1* signal to Notch transmembrane receptors on adjacent cells within the sensory primordium. One cell begins to express higher levels of *Delta1*, making it a nascent hair cell. This causes the upregulation of *Notch* expression in neighbouring cells which in turn inhibits further *Delta1* expression in these cells and destines them to become supporting cells. This mechanism of lateral inhibition allows the development of the initially homogenous cell population into the precise mosaic of hair cells and supporting hair cells seen in the fully developed sensory patches. Expression studies in the cochlea have shown that *Delta* is expressed from E13.5 initially in a single row corresponding to the inner hair cells, indicating that these may be specified slightly earlier than the outer hair cells, where *Delta* expression is seen from E16.5 (Morrison *et al.* 1999). By contrast *Jagged1* is initially expressed throughout the sensory primordium but as *Delta1* becomes upregulated in the nascent hair cells it becomes restricted to the supporting cells, making it another candidate for a role in lateral inhibition.

The mouse atonal homologue *Math1* has also been identified as a key player in the development of auditory hair cells. *Math1* knockout mice fail to develop

any AHCs (Bermingham *et al.* 1999), while overexpression leads to supernumerary hair cells (Zheng and Gao 2000). Unlike atonal which is expressed prior to the differentiation of the sensory primordium and is thought to play a role in its specification, *Math1* expression occurs after the sensory precursor domain has been established (Chen *et al.* 2002). This suggests that while not playing a role in hair cell precursor selection, *Math1* is essential for the early differentiation of hair cells.

The basic helix-loop-helix (bHLH) genes *Hes1* and *Hes5* (mammalian hairy and Enhancer-of-split homologs) are thought to play a role in hair cell production. They are expressed in the developing mouse cochlea from E14, with *Hes1* showing expression in the greater epithelial ridge (GER) and the lesser epithelial ridge (LER) while *Hes5* expression was seen most strongly in the supporting cells of the LER and in a narrow band of cells within the GER (Zine *et al.* 2001). Histological studies suggest that inner hair cells are derived from progenitor cells within the GER and outer hair cells from the LER (Lim and Rueda 1992; Zheng *et al.* 2000). In correlation with this, *Hes1* knockout mice showed an increase in the number of IHCs, whilst *Hes5* knockout mice had increased numbers of OHCs (Zine *et al.* 2001). In both cases the supernumary cells showed upregulation of *Math1*, indicating that *Hes1* and *Hes5* participate in hair cell formation via inhibition of *Math1*.

The cyclin dependent kinase inhibitor $p27^{Kip1}$ is expressed in the organ of Corti between E12 and E14, coinciding with the exit from the cell cycle (Chen and Segil 1999), and is localised to the supporting cells. Homozygous $p27^{Kip1}$ knockout mice possess supernumary AHCs but also retain SCs, implying a role in suppression of AHC development but not one in absolute differentiation. Interestingly, heterozygous knockout mice only develop additional IHCs, suggesting some form of dosage-dependent or time-dependent specificity.

A number of transcription factor genes have been implicated in the survival of hair cells late in embryonic development. When *Pou4f3* is knocked out the hair cells are specified normally but fail to mature and later degenerate (Erkman *et al.* 1996). Mutations in *Gfi1*, the mouse ortholog of the Drosophila gene senseless, give a very similar phenotype (Wallis *et al.* 2003) and expression of *Gfi1* is directly dependent on *Pou4f3* (Hertzano *et al.* 2004).

It is clear that although some of the mechanisms underlying hair cell fate specification and maturation have been elucidated, the process is far from being fully understood. The study of mouse mutants with phenotypes indicating that the process has been somehow disrupted is a useful approach to identifying further genes which may be involved, and the *bronx waltzer* mouse is one such mutant. None of the genes described above lie within the candidate region for *bronx waltzer*, and none give the same specific phenotype (see Section 1.4) when mutated or knocked out.

1.3 PATHOLOGY OF DEAFNESS

1.3.1 Pathology of the auditory system

Defects of the auditory system can be broadly grouped into two main types based on the region of the ear which is affected. Conductive defects occur when abnormalities in the outer or middle ears affect the transmission of sound to the inner ear. In the outer ear these may take the form of malformations which are severe enough to affect the conduction of sound. In the middle ear, abnormalities include disruption of the ossicular chain which impairs transmission of vibration to the oval window, or the condition of otosclerosis which causes progressive conductive hearing loss due to excess ossification.

The second class of auditory defect are those described as sensorineural, encompassing any anomalies in either cochlear function or neural responses. Neural defects may occur either in the peripheral neurons if the function or survival of primary inner ear neurons is affected, or in the central nervous system if processing of auditory information is impaired. Cochlear defects, which account for the vast majority of cases of genetic hearing impairment, can be further divided into three main classes which reflect different mechanisms of interference in the normal development and function of the ear. These three categories – morphogenetic, cochleosaccular and neuroepithelial defects – are discussed in the following paragraphs.

1.3.1.1 Morphogenetic defects

Morphogenetic defects are characterised by early disruption of the normal development of the inner ear, causing structural malformations which vary widely in severity. In some cases only the semicircular canals are affected, with defects ranging from a slight constriction in a single canal (most frequently the lateral canal) to the truncation of one or more canals such as in the mouse mutants with mutations in the *Chd7* gene on chromosome 4 (Bosman *et al.*

2005). In more severe cases, the inner ear can develop into a grossly misshapen cyst which is hardly reminiscent of the normal inner ear shape, such as in the *Dreher* mice (Bergstrom *et al.* 1999).

1.3.1.2 Cochleosaccular defects

Cochleosaccular defects are characterised by abnormality of the stria vascularis which tend to manifest in one of two ways. Either Reissner's membrane collapses onto the organ of Corti suggesting disruption of fluid homeostasis or there is a significant reduction in endocochlear potential, both of which imply strial malfunction and can lead to secondary hair cell degeneration. Many cochleosaccular abnormalities are accompanied by pigmentation defects caused by a lack of melanocytes. This lends support to the implication of the stria in such cases since melanocytes are present in the stria of the normal inner ear (Steel and Brown 1994) but are absent in the stria of some animals which lack a measurable endocochlear potential (Steel *et al.* 1987). Some cochleosaccular defects may be caused by defects in cell types other than melanocytes but these are less well understood.

1.3.1.3 Neuroepithelial defects

Neuroepithelial defects concern the sensory patches of the inner ear – the maculae and cristae in the vestibular system and the organ of Corti in the cochlea. The architecture of the sensory hair cells, and particularly the stereocilia on their surface, is vital to their function (see Section 1.1.2.1), so if their development or maintenance is disrupted then inner ear function can be severely impaired. Hair cell degeneration is often seen in mutants with neuroepithelial defects but this is likely to be a secondary effect of the mutation caused by a failure to be specified or develop or mature normally.

Neuroepithelial defects are thought to be the most common cause of human hearing impairment and as such represent an interesting area of investigation. This is because the identification of genes involved in hair cell development

and function hold the promise of a fuller understanding of the mechanisms of hearing. They may even provide the possibility of regenerating lost hair cells in an effort to restore hearing in the future. In addition, the majority of these defects manifest as autosomal recessive traits with a uniform penetrance and symmetry between the two ears, making them good models for the most common mode of transmission of genetic deafness in humans, that of autosomal recessive inheritance.

1.3.2 The mouse as a model in hearing research

The study of deafness in humans is difficult, with the usual complications of human pedigrees compounded by the fact that the ear is for the most part inaccessible, making detailed clinical analysis challenging. Gross deformities may be identified by means of computed tomography (CT) scanning, but the more common sensorineural forms of hearing impairment cannot be analysed in this way. Thus a model system is required in order to facilitate the study of auditory pathologies.

The mouse lends itself well as a model organism, being small in size, relatively easy to maintain in the laboratory and similar to man in terms of developmental and biochemical pathways, physiology and genetic conservation. Mice are particularly suited to genetic studies since they have a short generation time and can produce large litters, facilitating the construction of high resolution genetic maps. Crucially, the auditory and vestibular systems of mice and men are extremely similar. With the exception of the shape of the pinna/auricle, the structure of the mouse inner ear is almost identical to that of humans. These factors make the mouse an ideal model for the study of auditory physiology and pathology.

1.4 THE BRONX WALTZER (*bv*) MOUSE MUTANT

Bronx waltzer (*bv*) is an autosomal recessive mouse mutation causing abnormalities in the inner ear which result in mutant mice having deficiencies in both the auditory and vestibular systems. It takes its name from the place of its discovery – the Albert Einstein College of Medicine in the Bronx, New York – and the circling behaviour of mice homozygous for the mutant gene which has been described as "waltzing".

The mutation arose spontaneously on an unknown genetic background and was first described by Deol and Gluecksohn-Waelsch (1979). They examined sectioned mutant cochleas under the light microscope and found that the great majority of the inner hair cells (IHCs) were absent and that those which were present were almost all of abnormal appearance, while the outer hair cells (OHCs) seemed morphologically normal. They also noted that the neurones in the spiral ganglion underwent a rapid process of degeneration.

Following these initial observations it was felt that the *bronx waltzer* mouse represented an interesting model for hereditary deafness and for the development and function of the inner ear. As a result many studies have since been carried out in order to further characterise the mutation and the findings of some of these are outlined in the ensuing paragraphs.

1.4.1 Electrophysiology of bronx waltzer

1.4.1.1 Round window response recordings

Using round window response recordings in mice, three different responses can be determined.

The compound action potential (CAP) provides information concerning the activity of the auditory nerve. Since these signals originate from the inner hair cells, the CAP can be taken to be an indication of IHC function. To produce

a recordable signal it is expected that the IHCs are capable of afferent innervation and additionally that their response to sound is synchronous (Steel and Harvey 1992). In *bronx waltzer* the CAP was shown to be very small or absent in homozygotes (Bock *et al.* 1982), possibly a result of the reduced numbers of IHCs seen in the mutant cochlea and/or the reduction in the number of spiral neurons innervating the cells. In heterozygous mice the CAP thresholds were much higher than control mice at 10 kHz. This led to the suggestion that the mutated *bronx waltzer* gene may not be fully recessive in its effect on hearing.

Cochlear microphonics (CM) are thought to be an indicator of OHC function. They take the form of an alternating current response which mimics the waveform of the stimulus and a normal response indicates functional OHCs. In *bronx waltzer* homozygotes CM were present but greatly reduced when compared to wild type responses. As described later in this chapter, ultrastructural studies have shown that the OHCs in *bronx waltzer* mice appear normal, so these CM results are somewhat unexpected. Bock and Yates (1982) suggested that OHC function may be dependent upon IHC function, which is impaired in *bronx waltzer*. It is also possible that the OHCs are affected by the mutation in some way not visible by electron microscopy. Lenoir and Pujol (1984) observed an abnormal abundance of smooth endoplasmic reticulum (ER) which they speculated may be an indication of metabolic problems, although they did not study any control mice. In contrast, Takeno *et al.* (1994) studied CM amplitudes in chinchilla cochleae with selective IHC loss and found them to be close to normal. They also reported that under the light microscope (LM) the OHCs appeared normal and proposed that the IHCs and OHCs work independently of each other in the cochlear transduction process.

Summating potentials (SP) are offsets in the response sustained for the duration of the stimulus. Their polarity can be either negative or positive and they are thought to represent the intracellular direct current (dc) response of

both inner and outer hair cells (Steel and Harvey 1992). Bock *et al.* (1982) recorded both positive and negative summating potentials in *bronx waltzer* and in control CBA mice. Positive SPs were found to be reduced in the mutants but negative SPs were comparable with controls except at low frequencies. It was suggested that the OHCs which appear normal in the mutant are capable of producing both positive and negative dc potentials, although summating potentials are not well understood and are therefore difficult to interpret.

1.4.1.2 Auditory brainstem recordings

Auditory brainstem responses (ABRs) are recorded using differential pin electrodes situated beneath the skin at the vertex of the mouse head behind the pinna on the side to be recorded. The response is recorded as the difference between the signals from the two electrodes when a stimulus is presented and consists of several waves. It is thought that the first negative wave of deflection represents the neuronal activity of the cochlear nerve, and that the later waves reflect the functioning of the central auditory pathway. Schrott *et al.* (1989) found ABRs in bronx waltzer mice to be elevated by ~20dB SPL compared to CBA controls, a surprisingly small difference. It was suggested that the surviving IHCs, being morphologically intact and evenly spread throughout the length of the cochlea, provide a sufficient response so that the ABRs are relatively unaffected. In addition, since the OHCs appear normal, it is possible that their amplification of the basilar membrane movements enhances the stimulation of those IHCs which are present.

1.4.1.3 Otoacoustic emissions

Spontaneous emissions are recordable in a functioning ear canal but may also be associated with OHC loss. Conversely, evoked emissions are detectable in response to acoustic stimuli and include distortion product otoacoustic emissions (DPOEs). These can be recorded in both humans and rodents and are thought to reflect OHC activity. Studies of DPOEs in *bronx waltzer*

(Schrott *et al.* 1991) showed markedly raised thresholds, a surprising finding since the OHCs appear morphologically normal. Possible explanations of this result are that the OHC row disorganisation affects DPOEs, or that DPOEs rely on there being sensory input into the nervous system from functioning IHCs.

1.4.2 Ultrastructural studies of the bronx waltzer inner ear

As has been previously mentioned, the *bronx waltzer* mutant mice exhibit circling behaviour which is consistent with abnormalities in the vestibular system. They are also almost completely deaf, indicating problems with the auditory system which have been shown to be located in the cochlea (Deol and Gluecksohn-Waelsch 1979). Hence the structural studies of *bronx waltzer* mice have focussed on these two areas of the ear.

1.4.2.1 Ultrastructural studies of the cochlea

Following on from the studies of Deol and Gluecksohn-Waelsch, Lenoir and Pujol (1984) conducted a scanning electron microscopy (SEM) study of the cochleas of postnatal *bronx waltzer* mice. At birth, they found that the IHCs appeared to be either absent completely, rounded with the absence of stereocilia, or of normal appearance. At postnatal day P9 approximately 20-25% of the IHCs appeared normal, while the rest were absent or degenerated. It has also been noted that the pillar cells which lie adjacent to the row of IHCs in the normal mouse ear were malformed or missing in *bronx waltzer* mice (Deol 1981) and this was confirmed by Lenoir and Pujol who described disorganised pillar cells and OHCs in the region of the degenerated IHCs.

A more recent and detailed study of the *bronx waltzer* organ of Corti was carried out by Bussoli (1996) utilising scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The mice examined ranged in age from E16.5 to P30 and much information was gained pertaining to the series of events leading to the previously observed phenotype.

At E16.5 there was no evidence of hair cell formation in either control or *bronx waltzer* mice. At E17.5 the control mice showed the early stages of IHC development in the middle coil, while the mutant IHCs appeared to have shrunk to one third of their usual size and had taken on a rounded appearance. At E18.5 stereocilia could be seen to be differentiating in the control mice, initially in the basal end and followed by differentiation in the middle coil and apex as would be expected. The *bronx waltzer* IHCs in contrast now had a disorganised appearance and few showed signs of developing stereocilia. TEM at E19.5 also revealed vacuolation and nuclear condensation with rounded mitochondria, observations which are consistent with controlled cell death.

During the first few days after birth – P0 and P1 – the affected IHCs in the mutant mice were seen to be reabsorbed into the neuroepithelium. Following this event the OHCs start to become disorganised, although their individual appearance remains normal. From this stage onwards the observations were of depleted IHCs, with many missing or degenerated and a few appearing relatively normal. The disorganisation occasionally seen in OHCs is attributed to the disruption to the row of pillar cells caused by missing IHCs (see Figure 1.8). Occasionally atypical cells similar in appearance to OHCs were observed in positions normally occupied by IHCs.

In summary, it was found that the IHCs in *bronx waltzer* mice degenerate from E17.5, soon after their development would be expected to begin. It is possible that this process is due to a form of controlled cell death and it appears to lead after birth to disorganisation of the OHCs and pillar cells. The pattern of degeneration follows the pattern of normal hair cell differentiation in the organ of Corti, that is, from the basal end of the cochlea progressing towards the apex.

1.4.2.2 Ultrastructural studies of the vestibular system

The sensory patches of the vestibular system in *bronx waltzer* mice were first examined using light microscopy by Deol (1981) who found that the hair cells in both the cristae and the saccule were markedly reduced, had a rounded appearance and possessed malformed stereocilia. In the maculae the appearance of the hair cells degenerates rapidly after birth so that at P12 the saccular macula has none visible. In the utricular macula the same process occurs but less quickly. In the cristae more of the hair cells survive to adulthood, but many of these are degenerated. The vestibular ganglion has also been seen to exhibit some loss of cells.

Dêmemes and Sans (1985) carried out postnatal SEM and TEM studies of the cristae, utricular macula and vestibular ganglion. The first stage studied was P3, at which point the hair cells in the sensory patches already showed considerable signs of degeneration. Many cells had a bulging appearance and lacked stereocilia and ciliary tufts. The stereocilia which were present had an unusual morphology, being at varying stages of development and never attaining the normal staircase arrangement. By adulthood there is sometimes a complete absence of sensory cells and it was observed that those which remain resemble type II cells, but could in fact be developing type I cells which pass though a developmental stage similar to type II cells. It was therefore proposed that the *bronx waltzer* mutation may cause a halt to vestibular hair cell differentiation.

In order to understand the process leading up to the degeneration of the hair cells in the vestibular system, Cheong (2000) carried out a prenatal SEM study of the utricular macula and the superior crista (Figure 1.8). Observations made in these two sensory patches were extrapolated to include the saccular macula and the posterior and lateral cristae as the mutation has previously been shown to affect these regions in the same way (Deol 1981; Dememes and Sans 1985).

Control and *bronx waltzer* maculae and cristae were studied qualitatively at 4 stages of development – at E17.5, E19.5, P0 and P2. Some degeneration of the hair cells was observed at E17.5, indicating that the effect of the *bronx waltzer* mutation may be manifested in the vestibular system at an earlier stage than was studied here. Indeed, the observation of depleted hair cells may not represent the first event in the action of the mutation and in order to determine this a different approach would need to be used as intracellular events are not visible using SEM. It was observed that the density of stereocilia-bearing hair cells decreases over time between E17.5 and P2. The vestibular hair cells have never been observed to mature beyond this stage, since they appear to die prior to the final steps in differentiation.

In comparisons between the two sensory patches studied it was found that the utricular macula had a greater prevalence of degenerated cells at each stage studied, suggesting that the mutation takes effect here prior to affecting the superior crista (Cheong and Steel 2002). As it is thought that the maculae develop before the cristae this also follows the reported temporal pattern of differentiation, lending support to the idea that the *bronx waltzer* mutation takes effect at the time of hair cell differentiation as proposed in the organ of Corti (Bussoli *et al.* 1997).

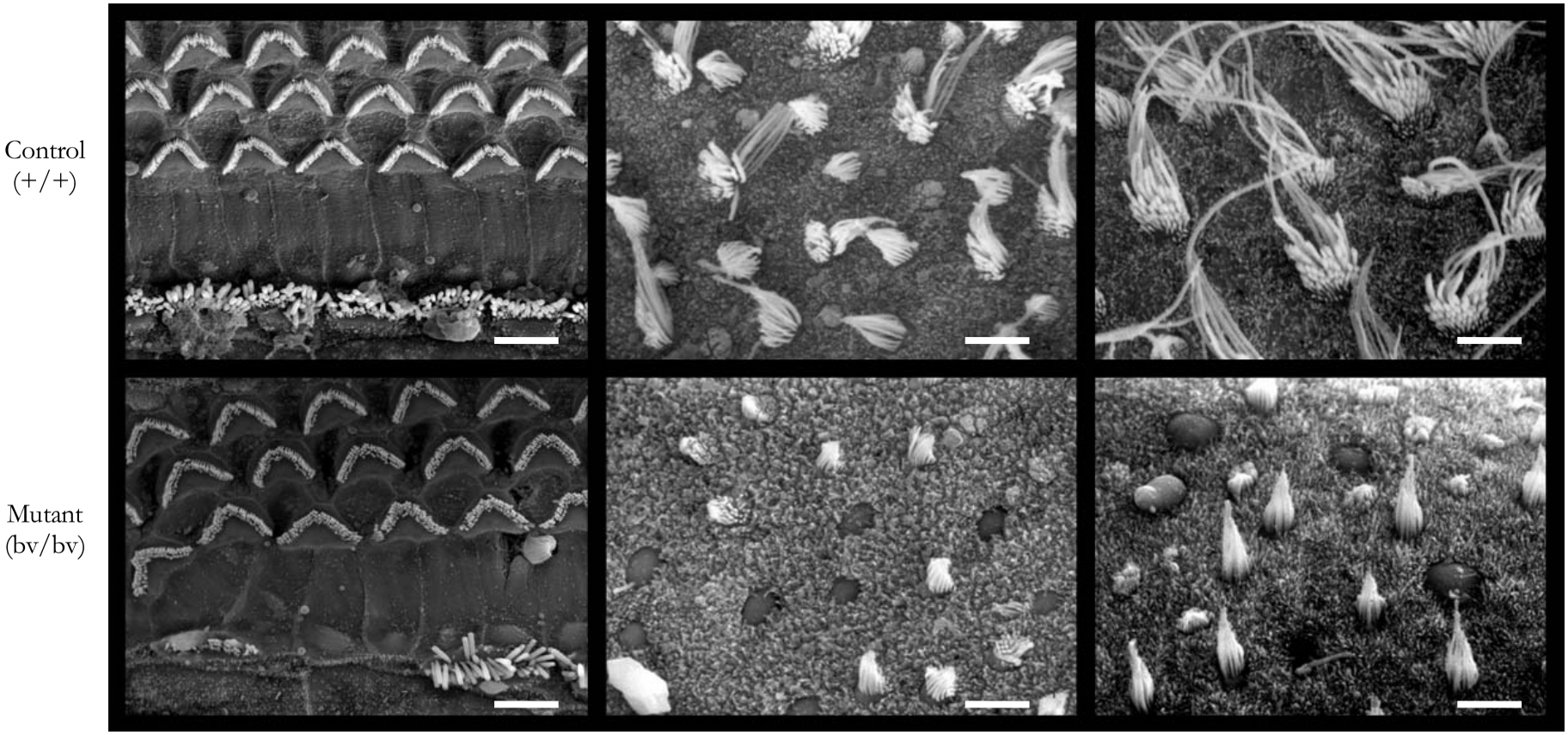

Figure 1.8: Scanning electron micrographs showing the apical surfaces of sensory patches in the wild type and *bronx waltzer* ear.
In the *bv* organ of Corti many inner hair cells are missing or degenerating. Outer hair cells appear normal, although their orientation is somewhat affected by the disorganised supporting cells. In the *bv* utricular macula and superior crista hair cells are underdeveloped with stereocilia much shorter than the wild type cells. In addition, many are seen to be in the process of being resorbed into the epithelium. Scale bar = 5μm (E19.5 images from Cheong, 2000).

1.4.3 Cochlear innervation in *bronx waltzer*

As previously mentioned, Deol and Gluecksohn-Waelsch (1979) reported a depletion in the cells of the spiral ganglion beginning at birth. However this finding was later contradicted by Lenoir and Pujol (1984) whose TEM study revealed no such degeneration.

Following on from these conflicting observations further studies have been made of the innervation of the cochlea, although the results are still somewhat contradictory. Observations made of the synapses at the bases of IHCs by Lenoir and Pujol (1984) found them to be present but abnormal, having no presynaptic densities and their afferent endings contained cytoplasm with a woolly appearance. Sobkowicz *et al.* (1988) found that surviving IHCs fail to be recognised by nerve fibres, and that the synapses with these IHCs are rarely formed. Schrott *et al.* (1989) obtained results which differed again, finding that remaining IHCs had synapses of normal appearance. It should be noted that these studies were carried out on *bronx waltzer* mice of differing genetic background and hence the variation in observations may possibly be attributed to the differing effects of modifier genes.

1.4.4 Previous mapping of the bronx waltzer mutation

1.4.4.1 Genetic mapping of the bv locus

A positional cloning approach was adopted to identify the *bronx waltzer* gene and an intraspecific backcross between the *bronx waltzer* mice and the mouse strain 101/H was established (Bussoli *et al.* 1997). This strain was chosen as a result of the higher degree of polymorphisms observed between it and the *bronx waltzer* background as compared with a number of other inbred strains. A mapping panel consisting of 1085 mice was established and used to position polymorphic markers in relation to the *bronx waltzer* locus. This work

led to the localisation of the *bronx waltzer* gene to a 1.86cM region of mouse chromosome 5, between flanking markers *D5Mit209* and *D5Mit188*.

Using the markers identified as being close to the *bv* locus and mapping these on a well characterised panel – the BXD recombinant inbred strains (Schadt *et al.* 2003) - it was possible to elucidate additional markers and previously identified genes which mapped to the same region of chromosome 5 and hence may represent candidate genes for *bronx waltzer*. In addition, the identification of nearby genes allowed comparison of the mouse map to the human map as many genes are conserved between the two species. Using this approach the candidacy of genes in the region was assessed by consideration of the role of the molecules they encode, but no obvious candidates were identified.

This work was continued by Cheong (2000), who refined the genetic map by mapping the flanking set of markers on two more recombinant panels – the BSS (Jackson Laboratory) and EUCIB (European Collaborative Interspecific Backcross; Breen *et al.* 1994) backcross panels. Once a region had been established on these panels by the placement of known markers, other polymorphic markers which had previously been mapped to the same region could be mapped back to the *bv*/101 backcross panel in the hope that they would map closer to the *bv* locus than the established flanking markers, thereby narrowing down the candidate region for the *bronx waltzer* gene. Unfortunately this was not the case. However, the further characterisation of the region in terms of markers, expressed sequence tags (ESTs) and genes allowed the identification of a region of conserved synteny on human chromosome 12q24.2. As the human sequence was then becoming available this provided a rich source of information about the possible constitution of the homologous mouse region, including the *bv* gene. The known genes from this region were assessed for their candidacy, but again no obvious candidates were identified.

1.4.4.2 Physical mapping of the *bv* locus

Cheong (2000) also carried out physical mapping in order to establish a contiguous set of clones across the candidate region. To accomplish this a yeast artificial chromosome (YAC) library was screened by PCR pool screening using the markers localised to the region. Clones identified as being positive for these markers were ordered according to the known order of the markers from the genetic map.

Using this method 2 YAC clones were identified which appeared to span the region between D5Mit25 and D5Mit209. However, these YACs displayed gaps in their STS content consistent with deletions or chimerism which are not uncommon in YACs, making them poor candidates for further study. Even so, the mapping of ESTs and STSs onto this physical map enabled the identification of further markers which were assessed as candidates for *bv*. None were considered particularly strong candidates.

1.5 THIS THESIS

At the outset of this work the *bronx waltzer* mutation had been localised to a 1.86cM region of mouse chromosome 5 using a mapping backcross consisting of 1085 mice. Of these there still remain 19 backcross mice exhibiting recombinations between the flanking markers. Therefore this backcross holds the potential to narrow the *bv* candidate region further if novel polymorphic markers can be found which map between the markers *D5Mit25* and *D5Mit209*. Hence the first aim of this work is to make use of the now readily available mouse genome sequence data as well as the facility to sequence large numbers of samples easily to identify new polymorphisms between bronx waltzer and the inbred strain used for the backcross, 101/H. This refinement of the critical interval should lead to a smaller number of candidate genes to be considered as causative agents for the mutation.

The second major aim of this project is to characterise the genes which localise to the *bronx waltzer* candidate region with the intention of identifying those most likely to give rise to the observed phenotype. The methods employed include an analysis of background literature and expression data, functional studies using the zebrafish as an alternative model and large-scale exon re-sequencing. The collation of these various forms of data regarding the candidate genes will allow them to be prioritised for further analysis, with the aim of discovering the gene which encodes *bronx waltzer* and thus further the understanding of its role in the ear.

Finally, since variability in the *bronx waltzer* phenotype has been reported on different genetic backgrounds, further investigations are to be carried out with the aim of identifying genes which may act as modifiers of the *bv* mutation. These could prove interesting since building up a picture of the molecules which interact with *bv* could aid in both its identification and subsequent characterisation.