An Exploration of the Role of Gap Junctions in the Avian Inner Ear

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Abstract

Gap junctions in the normal and regenerating inner ear of chick hatchlings have been immunohistochemically and functionally examined. Additionally, the effect of blocked gap junction channels on the proliferation of supporting cells in response to hair cell death has been assessed.

Studies have been conducted primarily in organotypic cultures of the auditory and vestibular sensory epithelium of chicken hatchlings. For regeneration studies, explants of basilar papilla and utricle were exposed to gentamicin, an ototoxic antibiotic, to induce hair cell loss. Following hair cell death, proliferation of supporting cells was upregulated and after 5 days immature, new hair cells were apparent. Blocking of gap junctions by carbenoxolone led to a significant reduction in the proliferation of supporting cells, suggesting an involvement of intercellular communication in the regeneration of hair cells.

Expression of connexin 43 (Cx43) and the chicken-specific connexin 31 (cCx31) was examined by immunohistochemistry and confocal microscopy. cCx31 was strongly expressed in the normal basilar papilla and utricle. Cx43 was confined to the supporting cells of the auditory sensory epithelium, where its immunolabelling co-localised with cCx31. In response to hair cell loss, Cx43 was transiently downregulated. This finding, together with the absence of Cx43 in the sensory epithelium of the utricle, which has a constant turnover of hair cells, might point to an inhibitory effect of Cx43 on supporting cell proliferation.

A dye-coupling assay, based on fluorescence recovery after photobleaching (FRAP), has been developed to examine the diffusion of the fluorescent tracer, calcein, between supporting cells in the intact tissue. Recovery of fluorescence occurred in supporting cells, but not in hair cells and was inhibited by the presence of carbenoxolone. Most notably, an asymmetric dye transfer across the basilar papilla was observed. The absence of directional permeability in the utricular macula, and in the drug-damaged basilar papilla, strongly suggests that the co-expression of cCx31 and Cx43 results in chemically rectifying gap junctions.
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I. INTRODUCTION

1. The Gap Junctions

With the advent of multicellular organisms, communication between cells became essential in order to coordinate responses to external stimuli, determine the function of individual cells within the context of the organism, and preserve the integrity of cellular networks. Appearing early in metazoan evolution, one mechanism for regulating the internal environment of a multicellular organism is direct intercellular communication via gap junctions.

Gap junctions are clusters of aqueous channels within the plasma membrane spanning the extracellular space between apposing cells. They mediate direct intercellular signalling by allowing the diffusion of ions, metabolites and second messengers up to a molecular weight of 1 kDa between the cytoplasms of adjacent cells (reviewed by Bruzzone et al., 1996; Kumar and Gilula, 1996; Evans and Martin, 2002; Nicholson, 2003). In mammals, virtually all cells form gap junctions at some point during their development, although they are absent in a few fully differentiated cells such as erythrocytes, skeletal myocytes, and spermatozoa. In tissues containing excitable cells, gap junctions permit the direct propagation of ionic currents at electrical synapses, thereby synchronising the firing rate of neurons or the muscle contractions in the heart, uterus or intestine. The spread of action potentials from cell to cell without the 1 ms delay inherent in chemical synapses is crucial for escape reflexes in invertebrates and cold-blooded vertebrates. Gap junctions in non-excitable tissue mediate in general the nutrient transfer between cells, maintain homeostasis by buffering the concentrations of ions and small metabolites, and play a role in intercellular signalling by propagating second messengers such as cyclic adenosine monophosphate (cAMP) and calcium ions (Ca^{2+}).

Evidence for direct intercellular communication came in the 1950s from electrophysiological studies of Purkinje fibres in the mammalian heart (Weidmann, 1952) and the motor synapses of the giant crayfish (Furshpan and Potter, 1959). The structure responsible for the electrical coupling of myocytes and neurons was characterised several years later by electron microscopy as a close apposition of the plasma membranes of two neighbouring cells separated only by a 2-4 nm gap (Robertson, 1963). X-ray diffraction analysis and electron microscopic studies revealed that this “gap” is bridged by two hexameric hemichannels that align in the extracellular space to form a complete intercellular channel (Figure I.1A, Makowski et al., 1977).
Gap junctions have been identified in every metazoan phylum, from cnidaria to chordata (Filshie and Flower, 1977; Becker et al., 1998). However, the functional conservation of these intercellular channels does not extend to their own molecular composition. In vertebrates, the hemichannels, known as connexons, are formed by the oligomerisation of transmembrane proteins, called connexins (Cx). In invertebrates, gap junctions are composed of innexins, a protein family with little sequence homology to connexins, but surprisingly similar topography and physiological properties (reviewed by Phelan and Starich, 2001). Recently a protein family homologous to innexins, called pannexin, has been identified in vertebrates (Panchin et al., 2000; Baranova et al., 2004) and been shown to constitute an additional class of electrical synapses within the vertebrate nervous system (Bruzzone et al., 2003).

Figure 1.1. The gap junction channel.
Model of gap junction channels based on (A) X-ray diffraction and electron microscopy and (B) electron crystallography. Bar = 2 nm. [Reproduced from Makowski et al., 1977 (A) and Unger et al., 1999 (B).]

1.1. The structure of gap junctions

In vertebrates, gap junctions appear as plaques of densely packed particles in freeze-fractured plasma membranes, which can extend from several nm² to several μm² (Goodenough and Revel, 1970). Within these plaques, the individual intercellular channels are arranged in an organised pattern with a centre-to-centre spacing of ~10 nm (Hoh et al., 1993). As shown by electron crystallography at a resolution of 7 Å, the pore of each hemichannel narrows down from ~4 nm at the cytoplasmic side to ~1.5 nm at the extracellular side of the plasma membrane and then widens to ~2.5 nm in the extracellular space where the two connexons form a tight seal (Unger et al., 1997; Unger et al., 1999). The three-dimensional reconstruction of electron diffraction images of a recombinant gap junction channel demonstrated the dodecameric nature of the intercellular channel at a maximum resolution of 7.5 Å (Figure 1.1B). Each hemichannel
contains 24 α-helices, which correspond to the four transmembrane domains of the six protein subunits, the connexins (Unger et al., 1999).

Connexins, which were first biochemically identified in 1986 (Kumar and Gilula, 1986; Paul, 1986), are encoded by a multigene family thought to have diverged early in vertebrate evolution by gene duplication (Bennett et al., 1994). Two alternative nomenclatures are currently in use to characterise these transmembrane proteins with molecular weights ranging from 25 to 62 kDa. The more commonly used is based on the predicted molecular mass (in kDa) of the connexin protein (e.g. Cx26, Cx43 etc.), while the other considers phylogenetic homologies based on gene structure, overall sequence homology and matching of specific sequence motifs. Two major connexin classes, α and β, and a minor γ-class are noted within this nomenclature.

Up to 21 connexin genes have been identified in the mouse and human genome (reviewed by Willecke et al., 2002) and orthologues have been characterised in several non-mammalian species including chicken, Xenopus and zebrafish. Most connexin genes show a relatively simple structure, with two exons separated by an intron of variable size. Exon1 contains the 5’-untranslated sequences, while exon2 consists of the complete coding region and the 3’-untranslated sequences. Several putative binding sites for transcription factors have been identified in the promoter regions of connexin genes, including TATA box, and Ap-1-, Ap-2-, and GATA-binding sites (reviewed by Saez et al., 2003). This transcriptional control of connexin genes accounts for the temporal and spatial differences in the expression of connexins during development and among different tissues. Connexins show a tissue-specific expression with many cells expressing more than one connexin isoform. The human epidermis, for example, expresses 10 different connexins, while connexin expression in the eye is confined to just Cx46 and Cx50. The expression of more than one connexin isoform in a cell may lead to a variety of different molecular compositions of gap junction channels.

Figure 1.2. Possible arrangements of connexins in a gap junction channel.
The hexameric connexons can be homomeric or heteromeric, i.e. they are composed of only one type or of multiple types of connexins, respectively (Fig. 1.2). Gap junction channels can be homotypic or heterotypic, i.e. they are composed of two identical connexons or different connexons in terms of their connexin composition and/or connexin stoichiometry. Finally, a gap junction plaque can be composed of solely homomeric connexons or a mixture of homomeric and/or heteromeric connexons with different stoichiometries and arrangements.

Each connexin is composed of four transmembrane domains (M1-M4), two extracellular (E1, E2) loops, one cytoplasmic loop (CL), an amino-terminus (NT) and a carboxy-terminus (CT) (Fig. 1.3).

Two of the four transmembrane α-helices, which anchor the channel in the membrane, have been shown to contribute to the lining of the aqueous pore (Unger et al., 1999). Although the precise identity is still a matter of debate, site-directed mutagenesis studies have indicated that the third transmembrane domain may be a major contributor to the pore lining (Skerrett et al., 2002). M1 and M3 also appear to be crucial for the assembly and the trafficking of the hemichannels, as revealed by functional studies of experimentally-induced and disease-related mutations (Martin et al., 2001; Marziano et al., 2003). Furthermore, the border region of M1/E1 is thought to house the voltage gate (Verselis et al., 1994; Qu and Dahl, 2002).

The extracellular loops are responsible for the docking of apposing hemichannels, whose compatibility is thought to be determined by E2 (White et al., 1995). They are characterised by the highly conserved arrangement of six cysteine residues, which form disulfide bonds between the loops (John and Revel, 1991) and have been shown to be crucial for the functioning of the channel (Dahl et al., 1992). Based on structural data
(Unger et al., 1999) and mutagenesis studies (Foote et al., 1998), the folding of the extracellular loops as anti-parallel β-sheets has been proposed.

The intracellular amino-terminus has been shown to be necessary for the trafficking of connexins and/or their insertion into the membrane (Martin et al., 2000). A conserved proline residue (G12) has been implicated in voltage-gating (Purnick et al., 2000), but may also play a role in connexin trafficking as mutations at this site in Cx26 and Cx32 lead to the accumulation of connexins in the cytoplasm (D'Andrea et al., 2002; Wang et al., 2004). In addition, a putative calmodulin-binding site, identified in Cx32, may be one of the determinants for connexin oligomerisation (Ahmad et al., 2001).

Connexins differ mainly in the sequence motifs and lengths of their cytoplasmic loops and their carboxy-termini. Such variations in the CL and the CT are thought to be the primary factors producing divergent functional properties of gap junctions, as those domains have been implicated in the regulation of gap junctions (reviewed by Harris, 2001; Saez et al., 2003). For example, differences in the primary sequences of CL and CT account for the different permeability and voltage sensitivities of the closely related isoforms Cx26 and Cx30 (Manthey et al., 2001).

The carboxy-terminus is the major site for the regulation of gap junctional communication as it contains consensus sites for various protein kinases as well as scaffolding and signalling proteins. Most connexins, with the exception of Cx26 (Traub et al., 1987), have been demonstrated to be phosphorylated on serine, tyrosine and threonine residues within the C-terminus. Phosphorylation has been implicated in the regulation of gap junctional communication through a number of mechanisms, including the biosynthesis, degradation, trafficking and assembly of connexins, and channel gating (reviewed by Lampe and Lau, 2000; Lampe and Lau, 2004). In addition, the C-terminus of some connexins contains postsynaptic density-95/discs large/zonula occludens-1 (PDZ) motifs, which are known to mediate protein-protein interactions (reviewed by Duffy et al., 2002; Thomas et al., 2002; Herve et al., 2004).

1.2. The formation and degradation of gap junctions

Like the majority of membrane proteins, connexins are synthesised, sorted, assembled and trafficked along the general intracellular transport route known as the secretory pathway (reviewed by Laird, 1996; Yeager et al., 1998; Falk, 2000; Segretain and Falk, 2004). Connexins are synthesised by membrane-bound ribosomes in the endoplasmic reticulum (ER) and acquire their final transmembrane topology during their co-translational integration into the ER membrane. On their transit from the ER
through the trans-Golgi network, the connexins oligomerise into hexameric connexons. The exact location of connexon assembly may differ between connexins and cell type, as the ER (Falk et al., 1997; Ahmad et al., 1999), the ER-Golgi intermediate compartment (Diez et al., 1999), and the trans-Golgi networks (Musil and Goodenough, 1993) have been indicated as possible sites for connexin oligomerisation. In addition to the secretory pathway, Golgi-independent trafficking routes have been shown for some connexins, such as Cx26 for which the direct insertion and oligomerisation into plasma membranes by a post-translational mechanism has been observed (Martin et al., 2001).

Immunoprecipitation analysis showed that connexin isoforms do not assemble in random order but interact selectively allowing only the formation of homomeric connexons or certain types of heteromeric connexons within the same classes (Falk et al., 1997). Indeed, every heteromeric hemichannel reported so far is composed of members of the same class, as exemplified by Cx43 (α1), which oligomerises with α-isoforms such as Cx37 (Brink et al., 1997) and Cx40 (He et al., 1999) but not with the β-isoforms Cx32 (Das Sarma et al., 2001) and Cx26 (Gemel et al., 2004).

Time-lapse microscopy of connexins labelled with green fluorescent protein (GFP) in conjunction with fluorescence recovery after photobleaching (FRAP) revealed that the assembled connexons are transported from the Golgi complex to the plasma membrane in vesicles along microtubules and are inserted predominantly into non-junctional membrane areas by fusion of the vesicles. The hemichannels then move laterally to the junctional site, where they are incorporated into the periphery of existing plaques (Lauf et al., 2002).

Non-junctional connexons remain in a predominately closed state to prevent the uncontrolled leakage and influx of ions and metabolites to and from the extracellular medium that would cause cell death. However, there is increasing evidence that hemichannels, independently of functional intercellular channels, play a role in intercellular signalling (reviewed by Goodenough and Paul, 2003). Open hemichannels have been implicated in a variety of functions, including the propagation of intercellular Ca²⁺ waves by the release of adenosine triphosphate (ATP) into the extracellular space, the prevention of apoptosis of osteocytes, and the inhibition of cones by horizontal cells in the retina that underlies the centre surround antagonism.

The docking of connexons is facilitated by calcium-dependent cell-adhesion molecules such as E-cadherin, which may provide the close apposition of the plasma membrane necessary for the interaction of the two apposing hemichannels (Jongen et al., 1991). The precise mechanisms of the docking are not known, but are likely to occur.
through non-covalent forces (Ghoshroy et al., 1995). To create a tight seal in the extracellular space, the hemicannels are staggered by 30° to allow the interdigitation of the extracellular loops of apposing connexons to form concentric double β-barrels (Foote et al., 1998).

The assembly of hemicannels into gap junction plaques is subject to the same compatibility checks as connexon oligomerisation and docking in that only connexons belonging to the same class assemble into heteromeric, and probably heterotypic, gap junction channels. Connexin isoforms of different classes, such as Cx26 (β2) and Cx43 (α1), assemble exclusively into homomeric and homotypic channels or segregate into distinct domains within the plaque (Falk, 2000). The expression of incompatible connexins results in the formation of communication compartments that are regarded important for certain intercellular signalling events, for example during pattern formation in the embryo (reviewed by Levin, 2002).

Surprisingly for a channel protein, connexins turn over very rapidly, with a half-life ranging from 1-5 hours (Saffitz et al., 2000). The removal of gap junctions from the plasma membrane occurs through the internalisation of complete, or large fragments, of gap junction plaques within double-membrane vesicular structures (Jordan et al., 2000). However, it has also been suggested that there is a mechanism which balances the removal of old channels from the centre of the plaque and the accretion of newly synthesised channels to the margin (Gaietta et al., 2002; Lauf et al., 2002). The complete gap junction degradation appears to involve both lysosomal pathways, which generally degrade endocytosed proteins, and proteosomal pathways, which are involved in the removal of cytosolic and nuclear proteins (reviewed by Berthoud et al., 2000; Berthoud et al., 2004). One of the signals for degradation may be the modification of connexins by ubiquitylation. In the mammalian inner ear, an ubiquitin ligase, the organ of Corti protein 1 (OCP1), has been shown to co-localise with Cx26 and may participate in the targeted degradation of connexins (Thalmann et al., 2003).

The dynamic formation and degradation of connexins is an indicator that the number, composition and properties of gap junctions are highly regulated. This allows the rapid modulation of gap junctional communication according to the physiological requirements of the cell, as exemplified by the acute increase in Cx43 in the myometrium prior to and during labour (Garfield et al., 1977). The post-translational control of the assembly, trafficking and degradation of gap junctions involves phosphorylation (reviewed by Lampe and Lau, 2000; Lampe and Lau, 2004) and interaction with other proteins (reviewed by Duffy et al., 2002; Thomas et al., 2002;...
Herve et al., 2004). For example, the phosphorylation of Cx43 by protein kinase C (PKC) has been reported to stimulate the removal of gap junctions from the plasma membrane, whereas cAMP increases the trafficking and assembly of gap junctions (Atkinson et al., 1995; Burghardt et al., 1995; Paulson et al., 2000). In addition, the direct interaction of Cx43 with ZO-1, a member of the protein family of membrane-associated guanylate kinases (MAGUK), has been implicated in the stabilisation of gap junction plaques (Toyofuku et al., 1998).

1.3. The permeability of gap junctions

The traditional characterisation of gap junctions as passive, non-specific passageways for molecules with a molecular weight of less than 1 kDa has been revised in recent years in the light of compelling evidence for a connexin-dependent modulation of intercellular communication. The expression of connexins in cell lines with minimal endogenous coupling, e.g. HeLa cells or Xenopus oocytes, enabled comparative studies of the relative permeability of different connexin isoforms using both electrophysiological measurements and intercellular diffusion of fluorescent dyes. These studies revealed a wide variation in the conductance, and the charge and size selectivity of gap junction channels, all of which were determined by the molecular identity of the connexins (reviewed by Harris, 2001; Goldberg et al., 2004).

The recording of single-channel currents of different homotypic channels using dual whole-cell patch clamping revealed a variety of unitary conductances ranging from \(~20\) pS to \(~300\) pS (Nicholson et al., 2000). Ion substitution experiments have shown that, consistent with a slight fixed negative charge in the pore (Brink and Dewey, 1980), most junctional channels have a preference for cations, which can vary up to 10 fold between different connexin isoforms (Veenstra, 1996). This charge selectivity is not correlated with the channel conductance, as exemplified by Cx32 that has a relatively small single channel conductance of 55 pS but shows almost no ionic preference (Veenstra, 1996; Suchyna et al., 1999). Although the differences in the ionic selectivity of connexin isoforms are relatively modest, it has nevertheless been postulated to be sufficient to produce rectification in certain heterotypic junctions (Suchyna et al., 1999).

The analysis of the intercellular diffusion of fluorescent dyes varying in size and charge, such as the negatively charged Lucifer yellow (443 Da, -2) and the positively charged Neurobiotin (287 Da, +1), has uncovered distinct limiting pore diameters, as well as charge preferences and molecular selectivity for different gap junction compositions (Elfang et al., 1995; Cao et al., 2002). These data show that the connexin
composition has a major impact on the discrimination of solutes with a size of ~1 nm. The exclusion limits of gap junctions vary from 0.7-1.5 nm (200-800 Da) and are not correlated to the channel conductance, as exemplified by Cx37, which has, despite having the largest unitary conductance (350 pS), a size-cut off around 0.7 nm (Gong and Nicholson, 2001).

Of direct biological significance is the dramatic selectivity for endogenous metabolites and second messengers. It is known that gap junctions are permeable for Ca$^{2+}$, inositol 1,4,5-trisphosphate (IP3), nucleotide triphosphates and cyclic monophosphates, amino acids, and glucose and its metabolites (reviewed by Harris, 2001). However, some connexin isoforms show preferences in the transfer of these signalling molecules, as has been demonstrated for adenosine, ATP, glutamate and glutathione (Goldberg et al., 1999; Goldberg et al., 2002), IP3 (Niessen et al., 2000) and cyclic nucleotides (Bevans et al., 1998). By comparing the relative permeability of Cx32 and Cx43 for $^{14}$C-labelled metabolites, Goldberg et al. showed that adenosine transfers 12 times better through channels formed by Cx32, while ATP transfers 300 times better through channels formed by Cx43. This suggests that the phosphorylation of adenosine can dramatically shift its permselectivity from Cx32 to Cx43. As ATP is negatively charged in respect to adenosine, Cx32 channels may restrict the transfer of negatively charged solutes more than Cx43 gap junctions. However, the selective transfer of molecules through gap junction channels may not be caused by differences in charge alone, as channels formed by Cx32 have a higher permeability for the anionic IP3 than Cx26 or Cx43 (Niessen et al., 2000).

It has been suggested that even small differences in the relative permeability of gap junctions could lead to significant differences in biological responses, if one considers the oscillatory changes and the limited half lives of the permeates (Harris, 2001). This modification is enhanced by the regulatory effects that some of the permeates, e.g. Ca$^{2+}$ and cAMP, have on channel gating. In addition, heterotypic/heteromeric junctions might amplify these differences further, as shown for Cx26/Cx32 junctions, in which the selectivity for the closely related cAMP and cGMP was determined by the stoichiometry and/or arrangement of Cx26 (Bevans et al., 1998).

1.4. The gating of gap junctions

Like conventional ion channels, gap junctions are gated, that is they undergo a reversible conformational change that opens and closes the channel in response to specific stimuli. Unusually for ion channels, however, they typically remain open at
rest, and only close in response to voltage (reviewed by Harris, 2001; Bukauskas and Verselis, 2004), and certain chemical stimuli (reviewed by Peracchia, 2004).

Most vertebrate gap junctions are sensitive to transjunctional voltage ($V_j$), i.e. they close when a potential difference between the coupled cells develops. In addition, some channels (Cx26, Cx30 and Cx43) are also sensitive to transmembrane voltage ($V_m$), i.e. the potential difference between the cytosol and the extracellular medium. $V_j$ gating is characterised by the rapid (1-2 ms) but incomplete closure of the channel and a slower transition (10-60 ms) from this subconductance state to a fully closed state, which is dependent on the hyperpolarisation of the cell. It has been suggested that the slow $V_j$ gate is responsible for maintaining uncoupled hemichannels in a closed state at physiological resting potentials (Bukauskas and Verselis, 2004). The function of the fast voltage gate is unclear, but a study of Cx43 and Cx46 hemichannels demonstrated that an activated voltage gate preferentially restricts the passage of fluorescent tracers, suggesting that the voltage gate allows electrical coupling while restricting the exchange of larger molecules with potential signalling properties (Qu and Dahl, 2002).

In homotypic junctions, the imposition of a voltage difference with either polarity results in a symmetrical decrease of the junctional currents over time. However, the polarity of the fast $V_j$ gate differs among the connexin isoforms, e.g. Cx26 closes with depolarisation while Cx32 closes with hyperpolarisation. Gap junctions comprising different connexin isoforms have therefore divergent properties of voltage dependence, varying in their polarity and voltage sensitivity, which can result in the asymmetric voltage dependence in certain heterotypic combinations (Barrio et al., 1991; Verselis et al., 1994; Bukauskas et al., 2002).

Two models for the closure of gap junction channels have been proposed: the blockage of the channel by the interaction of a gating particle with the pore (ball on a chain model) and the alteration of the pore size by the rotation and tilting of the connexins (iris model, Unwin and Ennis, 1984). Results from site-directed mutagenesis favour the "ball on a chain"-model for chemical gating, in which the carboxy-termini of the connexins would act as gating particles that swing around to interact with the intercellular loops (Morley et al., 1996).

In addition to voltage gating, gap junctions close in response to a number of stimuli that generally reflect the poor health of a cell, such as high intracellular acidity and calcium levels, thereby effectively isolating the cell and preventing damage from spreading in this way. For example, acute ischemia in the heart muscle leads to an increase in intracellular $Ca^{2+}$ and $H^+$ and promotes uncoupling of myocytes (Beardslee et al., 1996).
et al., 2000). It is thought that low intracellular pH affects gating via an increase in Ca^{2+}. In the absence of suitable Ca^{2+} binding sites within the connexin sequences, Ca^{2+} gating is likely to be mediated by the calcium–receptor protein, calmodulin (reviewed by Peracchia, 2004). Another possibility is that Ca^{2+} gating results from the activation of specific protein kinases and the subsequent phosphorylation of the connexin.

Gap junction channels also close in response to a number of physiological stimuli, typically associated with increased cell division. These include growth factors such as the epidermal growth factor (EGF) and the platelet-derived growth factor (PDGF), oncogene protein kinases such as v-Src and PKC, and mitosis-associated kinases such as cdc2. While many tumour promoters, oncogenes and growth factors inhibit gap junctional communication, some growth inhibitors (e.g. cAMP) and anticancer agents increase intercellular coupling and connexin expression (reviewed by Trosko and Ruch, 1998; Yamasaki et al., 1999).

1.5. The function of gap junctions

Studies of connexin-deficient ("knockout") mice and human mutations have provided refined and often new information about the contribution of individual connexins to the function of gap junctions within their physiological context. The phenotypes of connexin knockout mice are generally associated with defects in the transfer of nutrients, tissue homeostasis, the synchronisation of electrical activity, and regulation and coordination of cellular interactions through intercellular signalling (reviewed by Simon and Goodenough, 1998; White and Paul, 1999; Kelsell et al., 2001; Willecke et al., 2002).

For example, the ablation of Cx40, an isoform expressed in cardiomyocytes, results in delayed impulse propagation in the conductive myocardium and atrial arrhythmias due to impaired synchronisation of the contraction of the cardiomyocytes through gap junctional communication (Kirchhoff et al., 1998). Female Cx37 knockout mice are infertile as the impaired coupling between oocytes and granulocytes suppresses follicle growth, oogenesis and ovulation (Simon et al., 1997). Cx43-deficient mice die at birth of asphyxiation as they are unable to oxygenate their blood discretely due to heart malformations that restrict the blood flow to the lungs (Reaume et al., 1995). This aberrant development of the heart might be due to retarded migration of neural crest cells (Lo et al., 1999). Cx32 knockout mice exhibit a 25-fold increased rate of spontaneous hepatic tumour formation (Temme et al., 1997), lending support to the long
standing hypothesis that the loss of gap junctional communication will lead to unsuppressed cell growth (Loewenstein and Rose, 1992).

Studies of knock-in mice, in which one connexin is exchanged for another, shed light on the functional specialisations of individual connexins. For example, the replacement of Cx43 with either Cx32 or Cx40 rescued the cardiac malformation of Cx43-deficient mice, pointing to a degree of redundancy among connexins during heart morphogenesis. However, the knock-in mice developed new defects that affected the mammary gland and testis, suggesting that certain functions cannot be compensated for by other connexins (Plum et al., 2000).

Another example of functional specialisation of connexins is provided in the lens of the eye, where Cx46 and Cx50 gap junctions have been implicated in the maintenance of homeostasis necessary to prevent precipitation of crystallins and cataract formation in lens fibres. While the deletion of both Cx46 and Cx50 lead to cataracts, the phenotypes of the two knockout mice differ. Cx46 knockout mice exhibit a severe, senile-type cataract (Gong et al., 1997), whereas Cx50 knockout mice develop a milder cataract but the overall size of the eye and lens is smaller than normal (White et al., 1998). Knock-in studies confirmed the different roles of the two isoforms, as the functional replacement of Cx50 with Cx46 corrected defects in cellular differentiation and prevented cataracts, but did not restore normal growth (White, 2002). These data show that the intrinsic properties of Cx50 are required for cellular growth, whereas non-specific restoration of communication by Cx46 maintains differentiation and lens clarity.

In humans, mutations in several connexin genes are associated with hereditary diseases (reviewed by White and Paul, 1999; Willecke et al., 2002). For example, Cx46 and Cx50 mutations have been implicated in congenital cataracts, while mutations in genes encoding Cx31 and Cx30.3 can cause erythrokeratodermia variabilis. Mutations in Cx32, of which over 200 have been identified, are associated with the X-linked form of Charcot-Marie-Tooth disease (CMTX), a progressive neuropathy resulting in the degeneration of peripheral nerves, and characterised by distal muscle atrophy (Bergoffen et al., 1993). Cx32 is expressed in Schwann cells, which are wrapped around the axons of peripheral nerves. It forms intracellular (reflexive) gap junctions between adjacent myelin layers, thereby providing a shortcut for the diffusion of metabolites between the adaxonal layer and the cell nucleus in the outer layer. Mutations of Cx32, which result in the absence of functional channels or affect channel gating, impair the diffusion of metabolites through these reflexive gap junctions, causing the degeneration of the myelin sheets (Ressot and Bruzzone, 2000).
Recessive mutations in the human Cx26 gene are responsible for 50% of all cases of non-syndromic hearing loss (Rabionet et al., 2002). (Cx26 knockout mice die around embryonic day 11 due to the impaired transfer of glucose and other metabolites from the maternal to the foetal blood across the two trophoblast cell layers of the placenta (Gabriel et al., 1998). As humans contain only one cell layer, mutations in Cx26 do not lead to embryonic lethality.) In addition, several dominantly inherited mutations of Cx26 and Cx30, which are associated with hearing impairment, may occur in syndromes, such as Vohwinkel, that involve skin disorders (reviewed by Kelsell et al., 2001; Rabionet et al., 2002).

2. Gap Junctions in the Inner Ear

2.1. General anatomy and physiology of the mammalian inner ear

The inner ear of mammals is housed in the petrous portion of the temporal bone and is composed of an intricate series of canals that can be divided into three parts – the cochlear duct that houses the hearing organ, and the vestibule and semicircular canals that contain the organs of balance. The bony labyrinth is filled with perilymph, similar in ionic composition to other extracellular fluids, being high in sodium ions (Na\(^+\) = 140-150 mM) and low in potassium ions (K\(^+\) = 4-8 mM). Surrounded by perilymph and generally following the shape of the bony labyrinth is the membranous labyrinth. Its epithelial tubes are filled with endolymph, which, being rich in K\(^+\) (about 150 mM) and poor in Na\(^+\) (about 2 mM), more resembles an intracellular fluid.

The membranous labyrinth of mammals contains six sensory epithelia, which detect sound and head motion (Fig. 1.4). The cristaee of the three semicircular canals are sensitive to angular acceleration, the maculae of saccule and utricle in the vestibule respond to linear acceleration and head tilt, and the organ of Corti in the cochlear duct detects sound. Each epithelium contains two types of sensory receptors, known as hair cells, which are surrounded by non-sensory supporting cells (reviewed by Correia, 2002). The apices of hair and supporting cells are joined by tight junctions to form the reticular lamina, which acts as a diffusion barrier between the endolymph covering the apical surfaces of these cells and the perilymph surrounding their somata.

In the organ of Corti, a single row of inner hair cells (IHC) receives 95% of the afferent neurons of the cochlear portion of the VIII\(^{th}\) nerve. They are therefore regarded as the primary sensory cells, as they transmit acoustic information encoded in electrical
four rows parallel to the inner hair cells, are innervated predominately by efferent terminals and are thus under the influence of the central nervous system. Rather than transmitting acoustic information, outer hair cells enhance the sensitivity of inner hair cells and resonant behaviour of the cochlea by actively contracting and relaxing. This somatic contractility is thought to be the basis for the “cochlear amplifier”, which accounts for the nonlinearity of the cochlea, essential for the sensitivity and frequency discrimination of the cochlea (reviewed by Ashmore et al., 2000; Hudspeth et al., 2000). In the vestibular system, Type I and Type II hair cells are separated on a primarily morphological basis, but the functional significance of this distinction is still unclear.

Figure 1.4. The membranous labyrinth of the inner ear and its sensory epithelia.
Schematic illustrations of (A) the crista ampularis of the semicircular canals, (B) the utricular macula of the vestibule and (C) the organ of Corti of the cochlear duct. [Reproduced from Correia, 2002]

Hair cells derive their name from a bundle of modified microvilli or stereocilia at their apical surfaces that transduce mechanical stimuli into electrical responses. Up to 100 stereocilia, depending on the species and the location along the cochlear duct, are arranged in rows with increasing height, like the pipes of an organ. Between the rows, the apical tips of the stereocilia are connected with fine filaments, known as tip links,
which are thought to directly open a mechanoelectrical transduction channel when stretched (reviewed by Strassmaier and Gillespie, 2002). The hair bundles extend into special accessory structures, known as the tectorial membrane in the organ of Corti, the otoconial membrane in the maculae and the cupula in the cristae. The movement of the hair bundles relative to this extracellular matrix displaces the stereocilia, which if bent towards the tallest row causes the non-selective cation transduction channel to open. The subsequent influx of primarily potassium ions (K⁺) causes the depolarisation of the hair cell, which in turn opens voltage-sensitive Ca²⁺ channels. The resultant increase in intracellular Ca²⁺ triggers the release of neurotransmitters, most likely glutamate. This leads to the excitation of primary afferents and the increase of their firing rate above the spontaneous level. Conversely, when the stereocilia are bent away from the tallest row, the cell hyperpolarises, the transmitter release is suppressed, and the firing rate is inhibited. Following depolarisation, the membrane potential subsequently repolarises owing to the efflux of K⁺ through Ca²⁺ activated K⁺ channels located at the basolateral membrane of the hair cells. K⁺ then moves along an electrochemical gradient towards the connective tissue of the inner ear, where it is taken up by the fibrocytes of the spiral ligament or the vestibular connective tissue and secreted back into the endolymph by the marginal cells of the stria vascularis or the vestibular dark cells (reviewed by Wangemann, 1995; Wangemann, 2002a). In other words, K⁺ cycles between the fluid compartments of the inner ear.

One consequence of K⁺ cycling is the generation of the endocochlear potential (EP) in the organ of Corti, which is essential for the auditory function of the mammalian inner ear. Because of its particular ionic composition, the endolymph is charged positively (+80 mV) with respect to the perilymphatic space. This potential difference creates an electromotive force of 140 mV with respect to the resting potential of a hair cell (~ -60 mV) that drives K⁺ through the open transduction channels into the hair cells, irrespective of the high cytoplasmic K⁺ concentration (von Békésy, 1952; Davis, 1965). In addition, the high positive potential of the endolymph is thought to be crucial for the active movement of outer hair cells (reviewed by Robles and Ruggero, 2001).

The EP is generated and maintained by the stria vascularis, a stratified ion-transporting epithelium that lines the lateral wall of the cochlear duct. The basal and marginal cells enclose the interstrial space, which is isolated from the perilymph and endolymph by tight junctions between the basal cells and marginal cells, respectively. The intermediate cells, which are connected to the basal cells through gap junctions, protrude into the interstrial space and their KCNJ10-K⁺ channels are responsible for the
EP generation. Thus the endochoclear potential is essentially a diffusion potential, as it is generated in conjunction with a low K⁺ concentration in the interstitial fluid space and a high K⁺ concentration in the cytosol of the intermediate cells (reviewed by Wangemann, 2002a; Wangemann, 2002b).

2.2. Gap junctions in the mammalian inner ear

Gap junctions in the mammalian inner ear were first identified by freeze fracture in the mid 1970s among supporting cells of the organ of Corti (Jahnke, 1975; Gulley and Reese, 1976; Iurato et al., 1976), among supporting cells of the maculae of the vestibular system (Jahnke, 1975; Laciano et al., 1977), and among cells of the spiral ligament and stria vascularis (Jahnke, 1975; Reale et al., 1975).

Figure 1.5. Illustration of the gap junction systems within the mammalian cochlea.

The grey areas indicate the epithelial gap junction system, while the black areas mark the gap junction network within the connective tissue of the cochlea. [Reproduced from Kikuchi et al, 1995]

Based on ultrastructural studies, two gap junction networks - the gap junction system of the epithelial tissue and that of the connective tissue - have been described within the mammalian cochlear duct (Kikuchi et al., 1995) and the vestibular system (Kikuchi et al., 1994). The epithelial gap junction system of the cochlea connects the interdental cells of the spiral limbus, the inner and outer sulcus cells, the supporting cells of the organ of Corti, and the cells within the root processes of the spiral ligament (Fig. 1.5). In the connective tissue of the cochlear duct, gap junctions are present between the fibrocytes of the spiral limbus and the spiral ligament, the basal and intermediate cells of the stria vascularis, and the mesenchymal cells that line the scala
vestibuli. No gap junctions have been identified between the hair cells and the supporting cells in the organ of Corti and between the intermediate and the marginal cells of the stria vascularis, nor between adjacent marginal cells (Forge, 1984; Kikuchi et al., 1995; Forge et al., 2003a).

Within the vestibular system, the epithelial gap junction network of each sensory epithelium consists of supporting cells and transitional cells and, in the case of the crista ampulla, the cuboidal cells of the planum semilunatum. The gap junction system of the vestibular connective tissue comprises the subepithelial fibrocytes, the melanocytes beneath the dark cell region, and the mesenchymal cells that line the vestibule and are connected with the mesenchymal cells lining the scala vestibuli of the basal cochlea (Fig. 1.6). No gap junctions have been detected between hair cells and supporting cells, nor among dark cells (Kikuchi et al., 1994; Forge et al., 2003a).

![Figure 1.6. Illustration of the gap junction systems within the mammalian inner ear.](image)

The grey areas indicate the epithelial gap junction system, while the black areas mark the gap junction network within the connective tissue of the membranous labyrinth. TC, transitional cells; DC, dark cells; PS, planum semilunatum. [Reproduced from Kikuchi et al., 1994.]

2.2.1. Connexin expression

To date, the expression of a total of 16 connexin isoforms has been investigated in the mature mammalian inner ear, mainly by immunohistochemistry, RT-PCR and cDNA-macroarray hybridisation. Immunohistochemistry has revealed the expression of Cx26, Cx30, Cx31 and Cx43 in the cochlear duct of the adult mouse (Frenz and Van De Water, 2000; Xia et al., 2000; Ahmad et al., 2003; Forge et al., 2003b), and of Cx26,
Cx30 and Cx43 in the mature rat cochlea (Kikuchi et al., 1995; Lautermann et al., 1998; Suzuki et al., 2003) and the vestibular system of the mouse (Forge et al., 2003a). In addition, the mRNAs of Cx29 (Ahmad et al., 2003) and Cx50 (Forge et al., 2003a) have been detected in the mouse inner ear.

Of the four connexin-isotypes expressed in the inner ear, Cx26 and Cx30 are the predominant. Immunoreactivity for Cx26 was detected in all the cells comprising the epithelial and connective tissue gap junction systems of the cochlea and vestibule (Kikuchi et al., 1994; Kikuchi et al., 1995). Immunoreactivity for Cx30 has so far been detected in the supporting cells of the organ of Corti and the utricular macula, in the fibrocytes of the spiral ligament, and in the basal and intermediate cells of the stria vascularis (Lautermann et al., 1998; Forge et al., 2003a). Furthermore, double immunofluorescence revealed overlapping staining patterns for Cx26 and Cx30, suggesting a co-localisation of the two connexin isoforms within the gap junction plaques in the inner ear (Lautermann et al., 1998; Forge et al., 2002). This has been recently verified by co-immunoprecipitation of Cx26 and Cx30 (Ahmad et al., 2003; Forge et al., 2003a) and by immunogold labelling of thin sections, where Cx26 and Cx30 were evenly distributed within the gap junction plaques (Forge et al., 2003a).

In the mouse cochlea, Cx31 expression is confined to the fibrocytes of the spiral ligament, where it gradually decreases from base to apex (Xia et al., 2000; Forge et al., 2003a), and the auditory nerve (Lopez-Bigas et al., 2001). In the rat cochlea, weak immunostaining of Cx43 was detected between supporting cells in the organ of Corti (Lautermann et al., 1998; Suzuki et al., 2003). In the postnatal mouse, however, Cx43 is confined to cells lining the inside of the bony wall and the bone of the otic capsule, with no expression in the organ of Corti (Forge et al., 2003a; Cohen-Salmon et al., 2004). In the vestibular system, there is evidence for Cx43-positive gap junctions between the supporting cells of the sensory epithelium and the underlying connective tissue (Forge et al., 2003a).

2.2.2. Supporting cell coupling

The syncytial nature of supporting cells has been electrophysiologically confirmed in vivo (Oesterle and Dallos, 1990) and in vitro (Santos-Sacchi and Dallos, 1983). Corroborating ultrastructural evidence, no electrical coupling between hair cells and supporting cells could be detected.

Cell coupling in Hensen and Deiters' cells has been studied in detail by double patch clamp of isolated supporting cell-pairs (Santos-Sacchi, 1991; Nenov et al., 1998).
In accordance with the gating behaviour of gap junctions, supporting cell coupling is sensitive to transjunctional and transmembrane voltage, intracellular pH and Ca\(^{2+}\) (Sato and Santos-Sacchi, 1994), octanol (Santos-Sacchi, 1991), and free radicals (Todt et al., 1999; Todt et al., 2001). The predominant voltage-dependent currents in Hensen and Deiters’ cells are outward rectifying and selective for K\(^{+}\) (Santos-Sacchi, 1991; Nenov et al., 1998). It has been suggested that these outward K\(^{+}\) currents stabilise and restore the highly negative resting potential (-60 to -100 mV) of supporting cells after the depolarisation of the cell’s membrane potential caused by the accumulation of extracellular K\(^{+}\). Hensen cells display an additional inwardly rectifying current, which might help to buffer K\(^{+}\) levels in the extracellular spaces of the organ of Corti (Santos-Sacchi, 1991). A striking feature of supporting cell coupling is the diversity of voltage dependence and the directionality of voltage gating (Zhao, 2000; Zhao and Santos-Sacchi, 2000). Gap junctional conductances exhibit both symmetrical and asymmetrical voltage dependence, as well as none at all. The asymmetric voltage gating to the polarity of V\(_j\) or V\(_m\) could result in a directional current flow between the supporting cells. It has been suggested that this variability and directionality of gating is due to heteromeric or heterotypic gap junctions (Zhao and Santos-Sacchi, 2000).

These observations are consistent with the co-localisation of Cx26 and Cx30 within the gap junctions of the inner ear. Further support of the presence of Cx26/Cx30 channels comes from dye transfer studies. Following co-injection of Lucifer Yellow and Neurobiotin into individual supporting cells of the organ of Corti, Neurobiotin transferred to many cells, but Lucifer Yellow was retained in the injected cell (Forge et al., 2003b). This selectivity of dye transfer mirrors the dye spread in HeLa cells co-expressing Cx26 and Cx30 (Marziano et al., 2003). The particular arrangement of heteromeric and heterotypic Cx26/Cx30 gap junctions may thus account for two separate compartments within the organ of Corti that have been visualised by dye spread - one running from the pillar cell inwards and one running from the outer pillar cells outwards towards the lateral wall of the cochlea (Oberoi and Adams, 1998).

2.2.3. Role of gap junctions in the inner ear

Taken together, evidence from structural and functional studies supports the hypothesis that gap junctions play a role in maintaining the ionic homeostasis of the inner ear and provide the morphological substrate for potassium cycling within the inner ear, which is essential for the maintenance of the ionic composition of the endolymph
and the endocochlear potential in the cochlea (Kikuchi et al., 1995; Forge et al., 2002; Wangemann, 2002b).

One of the proposed pathways for K⁺ cycling in the cochlea starts with the entry of K⁺ into supporting cells, e.g. through the K-Cl co-transporter Kcc4 in Deiters' cells (Boettger et al., 2002). Once inside supporting cells, it may diffuse through gap junctions laterally to the lower part of the spiral ligament, where it is released into the extracellular space of the spiral ligament by root cells. Alternatively, K⁺ flows through perilymph towards the spiral ligament, where it is taken up by Type II fibrocytes via Na⁺/2Cl⁻/K⁺ co-transporter and Na⁺/K⁺-ATPase at their basolateral membranes. As Type II fibrocytes are part of the gap junction system that includes the strial basal and intermediate cells, K⁺ is able to pass through the tight junctional barrier of the basal cells of the stria vascularis. In the intermediate cells it is released via K⁺ channels into the intrastrial extracellular space, which is paralleled by the generation of the endochochlear potential. From the intrastrial space K⁺ is taken up by marginal cells via a Na⁺/2Cl⁻/K⁺ co-transporter and a Na⁺/K⁺-ATPase and then secreted back into the endolymph via K⁺ channels ( KCNQ1/KCNE1) in their apical membrane (reviewed by Wangemann, 2002a; Wangemann, 2002b).

Potassium cycling in the vestibular system is similar, though not associated with a large EP, as no equivalent to the intrastrial space is present. It is thought that fibrocytes connected by Cx26 may be involved in the delivery of K⁺ from the hair cells to the dark cells. Dark cells are physiologically and morphologically equivalent to the strial marginal cells in the cochlea. Their basolateral surfaces are directly exposed to the perilymph of the extracellular space, from which K⁺ is taken up by a Na⁺/K⁺-ATPase (reviewed by Wangemann, 1995).

2.3. Pathology of connexin-deficient mice

The deletions of Cx26 and Cx30 in the inner ear of mice result in severe hearing impairment (between 30-100dB) shortly after the onset of hearing but no vestibular dysfunction. This resembles the clinical phenotype of connexin mutations in humans.

In the mouse model for recessive Cx26-related deafness, the neonatal lethality of Cx26 knockout mice was overcome by restricting the deletion of Cx26 to the epithelial gap junction system (Cohen-Salmon et al., 2002). The inner ear of homozygous mice developed normally, but by postnatal day 14, soon after the hearing onset, apoptosis of supporting cells of the inner hair cells (IHC) was observed, extending later to the outer hair cells and their supporting cells. Cohen-Salmon et al. suggested that the death of
IHC-supporting cells is caused by oxidative stress due to the interference of accumulated \( K^+ \) with the removal of the neurotransmitter glutamate from the extracellular space. In addition, adult mutant mice showed a decreased EP and a lowered endolymphatic \( K^+ \) concentration, which is likely to have resulted from the mixture of endolymph and perilymph due to the disruption of the reticular lamina.

Another mouse model inhibits the function of Cx26 by expressing the Cx26 mutation R75W, which is associated with dominantly inherited hearing loss (Kudo et al., 2003). The dominant-negative effect of this missense mutation on the function of gap junction channels was confirmed by electrophysiology and dye transfer analyses that demonstrated that gap junctional communication was not only inhibited through homotypic R75W channels but also through R75W gap junctions containing wild-type Cx26 or Cx30 (Richard et al., 1998; Marziano et al., 2003). At 2 weeks of age, R75W mutant animals displayed deformities of both the tunnel of Corti and the supporting cells. By 7 weeks, the outer hair cells had degenerated, whereas the inner hair cells were still present. No apparent effect was detected in the stria vascularis, which was confirmed by a normal endocochlear potential. These findings suggest that the R75W mutation in Cx26 exerts its effects within the epithelial gap junction system of the inner ear by disruption of homeostasis of the cortilymph due to impaired K+ buffering.

The knockout of Cx30 in both the sensory epithelium and the connective tissues of the inner ear resulted in the failure to generate an endocholceral potential in two-week old homozygous mutant mice despite normal inner ear development and endolymphatic \( K^+ \) concentrations (Teubner et al., 2003). From postnatal day 18 onwards, the organ of Corti generally deteriorates, causing apoptosis of mainly inner and outer hair cells. In adult mice the endolymphatic \( K^+ \) concentration was decreased, contributing to the profound deafness of adult mutant mice.

The phenotypes of these transgenic mice demonstrate that Cx26 and Cx30 cannot compensate for each other. This supports the hypothesis that Cx26/Cx30 channels are uniquely suited to the physiological demands of the auditory organ and would account for the non-syndromic manifestations of Cx26 and Cx30 mutations, despite their expression in other tissues (Marziano et al., 2003; Forge et al., 2003a). The fact that the function of the vestibular system does not rely on a large endochoclear potential may account for the absence of vestibular defects despite the deletion of Cx26 and Cx30. In addition, the presence of Cx43 between supporting cells may compensate for the lack of Cx26 and Cx30 in K+ buffering.
2.4. Connexin mutations and hearing loss

The importance of gap junctions for auditory functions has been emphasized by the discoveries that mutations in genes coding for Cx26 (Kelsell et al., 1997), Cx30 (Grifa et al., 1999), Cx31 (Xia et al., 1998) and Cx43 (Liu et al., 2001) cause hereditary hearing loss. Connexin mutations are associated with autosomal recessive and dominant deafness, whose phenotypes are mostly confined to the inner ear (non-syndromic) but can occur with other clinical features (syndromic) (reviewed by Rabionet et al., 2002).

Despite the genetic heterogeneity of non-syndromic autosomal recessive deafness (DFNB), a single locus on chromosome 13q12, DFNB1, accounts for up to 50% of this type of hearing loss (Bitner-Glindzicz, 2002). The gene responsible for DFNB1 has been identified as \( \text{GJB2} \), which encodes Cx26 (Kelsell et al., 1997). More than 90 different mutations have been characterised in the \( \text{GJB2} \) gene, including splice, nonsense, missense and frameshift mutations, and deletions of one amino acid (see http://www.crg.es/deafness). With a carrier frequency of 1.5-2.5%, the most common mutation in European and North American populations is a deletion of a single guanine nucleotide, known as 35delG, which results in a frameshift and the subsequent premature termination of protein translation. Several rare missense mutations in \( \text{GJB2} \), which primarily affect amino acids within the first extracellular loop, have been detected in families with autosomal dominant inheritance (DFNA3). Such mutations cause progressive mild-to-profound hearing impairment and may be associated with various skin disorders including palmoplantar keratoderma and Vohwinkel syndrome.

A deletion in the gene coding for Cx30, \( \text{GJB6} \), has recently been linked to non-\( \text{GJB2} \)-related cases of deafness at the DFNB1 locus (del Castillo et al., 2002). In addition, a missense mutation in \( \text{GJB6} \) affecting the amino-terminal of Cx30 (T5M) is associated with autosomal dominant (DFNA3) middle to high frequency hearing impairment of late onset.

Mutations in the gene for Cx31, \( \text{GJB3} \), cause both non-syndromic autosomal recessive deafness (Liu et al., 2000) and non-syndromic, dominant, progressive hearing loss (DFNA2) that, consistent with the Cx31 expression pattern, affects preferentially high frequencies (Xia et al., 1998). In addition, a deletion of aspartic acid 66 located within the third transmembrane domain causes neuropathy accompanied by mild deafness (Lopez-Bigas et al., 2001). Several mutations in the gene encoding Cx32 (\( \text{GJB1} \)) lead to CMTX associated with mild hearing impairment. As no Cx32 is present in the auditory periphery (Forge et al., 2003a), the effects of the mutations are likely to occur via neuropathy in a similar way as postulated for Cx31. Finally, missense
mutations in *GJA1*, encoding Cx43, have been reported to cause autosomal recessive deafness (Liu et al., 2001).

2.5. **Aim of the study**

The severe phenotype of connexin mutations leaves no doubt about the importance of gap junctions for auditory function. Evidence gathered so far points strongly to the involvement of gap junctions in the maintenance of inner ear homeostasis. However it is reasonable to assume that this is not their only role in the vertebrate inner ear.

Gap junctions have been implicated in the coordination of cell proliferation and differentiation during development (Becker et al., 1999; Becker and Mobbs, 1999) and after injury (Goliger and Paul, 1995; Coutinho et al., 2003). Furthermore, the transformation of cells is accompanied by the loss of gap junctional communication, while the forced expression of connexins in neoplastic cells restores normal cell growth. This led to the proposal that gap junctions have a negative influence on cell growth and that certain connexins may act as tumour suppressors (reviewed by Trosko and Ruch, 1998; Yamasaki et al., 1999). It is therefore of interest to explore the possible involvement of gap junctions during hair cell regeneration.

Auditory and vestibular hair cells of vertebrates can be damaged as a consequence of acoustic trauma, exposure to certain antibiotics, infections, and ageing. In mammals, hair cell loss leads to permanent deficits in hearing and balance, as there is no postembryonic production of hair cells in the organ of Corti (Ruben, 1967) and only limited regeneration of damaged hair cells in the vestibular system (Forge et al., 1993; Walsh et al., 2000). In contrast, non-mammalian vertebrates are able to produce new hair cells throughout their adult lives in both the auditory and vestibular sensory epithelia and so replace any lost hair cells (reviewed by Corwin and Oberholtzer, 1997; Cotanche, 1999; Bermingham-McDonogh and Rubel, 2003).

Birds, as the only warm-blooded vertebrate able to regenerate their hair cells and possessing similar hearing and vestibular organs to mammals, are well established models to study hair cell regeneration in vertebrates. In response to hair cell loss, supporting cells in the otherwise mitotically quiescent avian cochlea enter the cell cycle, proliferate and subsequently differentiate into new, functional hair cells (Cotanche, 1999; Stone and Rubel, 2000). Like mammalian supporting cells, those of birds are extensively connected via gap junctions (Forge et al., 2003a). An inner ear-specific connexin, cCx31, has been identified in the chicken, which shares high sequence similarities to Cx26 and Cx30 (Heller et al., 1998). Otherwise little is known about the
composition and distribution of connexins in the avian inner ear and the functional coupling between supporting cells.

The aim of this project is therefore to characterise gap junctions in the normal and regenerating avian inner ear using immunohistochemistry and to examine their functional properties using fluorescence recovery after photobleaching (FRAP), in the hope that comparative studies of gap junctions of the inner ear of birds and mammals will shed light on the mechanisms that are fundamental for the working of the ear and that might underlie hair cell regeneration in the bird.
II. MATERIALS AND METHODS

1. Animals

Fertilized eggs of white leghorn chickens (*Gallus domesticus*) were obtained from a national supplier (Henry Stewart & Co Ltd, Lincolnshire) and incubated at 37.4°C in a humidified, rotating incubator (Octagon 20, Brinsea) for 21 days.

2. Organotypic cultures

Organotypic cultures of basilar papilla and utricular macula of hatchling chicks (embryonic day 21; E21) were used to study gap junctions in the sensory epithelia following hair cell loss and during hair cell regeneration.

The dissections were carried out under sterile conditions in a laminar flow hood using heat-sterilised instruments. In one continuous session 3 to 4 chicks were dissected as follows. The embryos were removed from the egg and decapitated, after the shells had been disinfected with 70% ethanol. The skinned heads were sagitally cut in half starting from the foramen magnum. The brains were removed and the beaks, jaws and crania were trimmed away from the bony casing of the inner ear and the skull pieces were placed in cooled Medium 199 with Hanks’ Salt and 25 mM HEPES (Invitrogen). Under a dissecting microscope the bone around the auditory and the vestibular nerves was carefully broken away with forceps to expose the saccular macula and utricular macula and the proximal half of basilar papilla. The cochlear duct was gripped at its very proximal tip with fine forceps and, by severing the nerve roots, carefully pulled out of the otic capsule. The utricular macula with parts of the membranous labyrinth was removed by grasping the distal end of the vestibular nerve trunk. The isolated epithelia, separated in left and right, were placed in fresh medium for further dissection. The nerve trunk, the otoconial membrane and the surrounding tissue were removed from the neuroepithelium of the utricular macula and its underlying connective tissue with fine forceps. The tegmentum vasculosum overlying the basilar papilla and the otoconia of the lagena macula were dissected away in cochlear ducts used for fluorescence recovery after photobleaching experiments.

The explants were transferred on a small spatula to membrane inserts (0.4 μm pore size, 30 mm diameter; Millicell-PCF, Millipore), placed in 6-well dishes (Nunc). Each insert contained up to 3 cochlear ducts and 3 utricular maculae, which were maintained in ~1.7 ml Medium 199 with Earle’s Salt, 25 mM HEPES, 26 mM sodium bicarbonate, 0.69 mM l-GLutamine and 10% horse serum (Invitrogen), as previously reported.
(Warchol and Corwin, 1993; Warchol and Corwin, 1996) and incubated in a humidified incubator in a 5% CO$_2$ atmosphere at 37°C for up to nine days. The culture medium was changed every other day by replacing 1 ml medium in the reservoir. To facilitate the attachment of the tissue to the PVP-free, polycarbonate membranes and ensure the supply of oxygen, the explants were only covered by a thin film of medium, accessing the medium in the dish through the microporous membrane.

2.1. Induction of hair cell loss

The ototoxic aminoglycoside gentamicin was used to induce hair cell loss in organotypic cultures of cochlear duct and utricular macula. After one day in culture, a set of explants was incubated in culture medium containing 1 mM gentamicin (Sigma) for 24 hours. The contralateral tissues served as controls and were maintained in standard culture medium. The following day gentamicin was washed off with fresh medium and the tissue was incubated in control medium for another 1, 3, 5, or 7 days. The explants were then fixed for 30 min with 4% paraformaldehyde (pH 7.6, PFA) in phosphate-buffered saline (PBS; Oxoid), followed by a thorough wash with PBS. To prevent bacterial contamination the fixed tissues were kept at 4°C in PBS containing 0.01% sodium azide (Sigma) until further processing.

2.2. Pharmacological blocking of gap junctions

To examine the effects of disrupted intercellular communication on cell proliferation following hair cell loss, explants were incubated with the gap junction blocker carbenoxolone (CBX; Sigma). Its inactive analogue glycyrrhizinic acid (GZA; Sigma) served as control. 1 mM stock solutions of CBX and GZA were prepared in Medium 199 with Earle’s Salt and used at a final concentration of 100 μM.

After one day in culture, some explants were incubated for a further 24 hours in culture medium containing 1 mM gentamicin, and either 100 μM CBX or GZA. Contralateral tissues, which served as undamaged controls, were maintained in culture medium containing either 100 μM CBX or GZA. After one day, gentamicin was washed off and the explants were incubated for another 1, 3 or 5 days in CBX or GZA supplemented medium, respectively (Fig. II.1). To monitor cell proliferation, 3 μM bromodeoxyuridine (BrdU; Sigma), a thymidine analogue that is incorporated into DNA during the S-phase of the cell cycle, was either continuously present in the medium or added 6 hours prior to fixation. BrdU-positive cells were subsequently detected by immunohistochemistry (see II.4.2).
3. Scanning electron microscopy

Scanning electron microscopy (SEM) was used to qualitatively assess hair cell loss and regeneration following gentamicin exposure. The samples were processed according to the thiocarbohydrazide-osmium tetroxide repeated (TOTO) method as previously described (Davies and Forge, 1987). All steps were carried out in processing baskets (Agar Scientific) at room temperature. Explants of the cochlear duct and utricular macula (without the otoconial membrane) were fixed 1, 3 and 5 days after gentamicin treatment in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 1 hour. After several washes with cacodylate buffer, the tegmentum vasculosum, the tectorial membrane and the otoconia of the lagena macula were removed and the tissues were postfixed in 1% buffered osmium tetroxide (OsO₄) for 1 hour. The tissues were then washed 6x5 min in distilled water, incubated for 20 min in a freshly filtered, saturated solution of thiocarbohydrazide (0.5% TCH, Sigma), washed again 6 times, and incubated in 1% aqueous OsO₄ for 1 hour. This procedure was repeated once and, after final washes in distilled water, the specimens were dehydrated in an ascending ethanol series (30%-50%-70%-90%-3x100%) for 10 min per step. The samples were transferred in 100% ethanol to a critical point dryer (Polaron) and dried with CO₂ using standard procedures. The specimens were mounted on aluminium stubs using a thin layer of conducting silver paint (Electrolube Ltd.) and then coated with gold/palladium in a sputter coater (S150B, Edwards) at 750 V and 40 mA for 1.5 min. The samples were examined in a scanning electron microscope (Jeol 5800) at 15 kV.
4. Immunohistochemistry

4.1. Connexin labelling

The reactivity of antibodies to the mammalian Cx26, Cx30, and Cx43 and to chicken Cx31 (see table II.1) in the auditory and vestibular sensory epithelium of hatchling chicks (E21) was examined in vivo and in vitro. HeLa cells transiently transfected with Cx26, Cx30 and cCx31 (see II.6.2) and stably transfected Cx43-HeLa cells (a kind gift from Prof. Nicholas Severs, Imperial College, London) served as positive controls. The primary antibodies were omitted in negative controls.

For in vivo preparations, basilar papillae and utricular macula were dissected in PBS as outlined above and immediately fixed in 4% PFA for 30 min. Some epithelia were fixed in 2% PFA to assess the influence of fixation on the affinity of the antibodies. Connexin expression was examined in wholemounts and cryosections. For wholemount preparations of the basilar papilla, the tegmentum vasculosum, tectorial membrane and the otoconia of the lagena macula were removed with fine forceps after the fixation. For cryosections, the fixed tissues were incubated in a 30% sucrose/PBS solution overnight at 4°C to prevent the formation of ice crystals. The following day, the tissues were embedded in a warm agarose/sucrose solution containing 1% low temperature gelling agarose (type VII), 18% sucrose, and 0.01% sodium azide (all Sigma) dissolved in PBS. The agarose was allowed to set and the dishes were stored at 4°C until use. Agarose blocks containing control and treated basilar papillae and/or utricular maculae were mounted with OCT compound (Tissue Tek, Sakura) on a cryostat holder and quickly frozen with Cryospray 134 (Bright Instruments). 15 μm thick serial sections were transversely cut at -25°C on a cryostat (CM1900, Leica). The sections were mounted on polylysine-coated slides (BDH), dried at 37°C for 1 hour and stored at -20°C until use.

Processing for immunohistochemistry was carried out in a moist atmosphere at room temperature on an orbital shaker, unless otherwise stated. The tissue sections and wholemounts, which were placed on multispot slides, were incubated for 1 hour in PBS containing 10% goat serum (GS, Sigma) and 0.2% Triton X-100 (TX-100, Sigma) to block non-specific sites and permeabilise the tissue. The epithelia were then simultaneously incubated in a polyclonal and a monoclonal anti-connexin antibody diluted in PBS containing 100 mM L-Lysine (Sigma) and 0.2% TX-100 (for dilutions see table II.1) overnight at 4°C. After a 30 min wash with 5 changes of PBS, the epithelia were incubated in the dark for 1 hour in Alexa Fluor 633-conjugated goat anti-rabbit IgG (Molecular Probes), fluorescein isothiocyanate (FITC)-conjugated goat anti-
<table>
<thead>
<tr>
<th>Connexin</th>
<th>Antibody</th>
<th>Type; Host</th>
<th>Peptide-Sequence</th>
<th>Position; Amino acids</th>
<th>Concentration</th>
<th>Source</th>
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</thead>
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<tr>
<td>Cx43 (α1)</td>
<td>C6219</td>
<td>Polyclonal; Rabbit</td>
<td>KPSSRASSRASSRPRPDDLEI</td>
<td>C-terminus; 363-382</td>
<td>1:400</td>
<td>Sigma</td>
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<td>MAB3067</td>
<td>Monoclonal; Mouse</td>
<td>TGPLSPSKDCGSPKYAYEN</td>
<td>C-terminus; 252-270</td>
<td>1:100</td>
<td>Chemicon</td>
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<tr>
<td>Gap 1</td>
<td>Monoclonal; Mouse</td>
<td>EIKKFKYGIEEHC</td>
<td>Cytoplasmic loop; 131-142</td>
<td>1:100</td>
<td>D. Becker, UCL, London</td>
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<tr>
<td>Cx26 (β2)</td>
<td>Gap 28H</td>
<td>Polyclonal; Rabbit</td>
<td>SEKFDIEEIKTQ</td>
<td>Cytoplasmic loop; 114-125</td>
<td>1:200</td>
<td>W.H. Evans, Univ. of Wales, Cardiff</td>
</tr>
<tr>
<td>71-0500</td>
<td>Polyclonal; Rabbit</td>
<td>SEKFDIEEIKTQ</td>
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<td>Zymed</td>
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<td>Cx30 (β3)</td>
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<td>71-200</td>
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<td>C-terminus; 215-229</td>
<td>1:100</td>
<td>produced by Sigma Genosys</td>
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Table II.1. Connexin-antibodies used for immunohistochemistry.
mouse-IgG (Pierce), and 1 μg/ml tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin (Molecular Probes) in PBS/Lys/TX-100. In cases where the staining of the nuclei was required, the tissues were incubated for another 5 min in 300 nM 4',6 diamidino-2-phenylindole dihydrochloride (DAPI). After 5 washes in PBS, the tissues were then mounted in anti-fade medium (Vectashield, Vectorlabs) and the coverslips were sealed with nail varnish. Hardened layers of nail varnish were used as supports for the cover slips to prevent the distortion of wholemount preparations.

The immunostained samples were examined by confocal laser microscopy (Leica TCS SP2) using 20x (HC PL APO CS 20x0.7 Dry UV), 40x (HCX PL APO CS 40x 1.25 Oil PH UV), or 63x (HCX PL APO CS 63x 1.32 Oil PH UV) objectives with numerical apertures of 0.7, 1.25, and 1.32, respectively. FITC (excitation/emission 494/518 nm), TRITC (ex/em 555/580 nm), Alexa Fluor 633 (ex/em 632/647 nm) and DAPI (ex/em 358/461 nm) were excited simultaneously with the 488, 568, 633, and 364 nm laser lines, respectively and their emission filtered through a prism spectrophotometer. Z-series of the tissues were taken at 1 μm intervals using triple channel scanning and were transformed into projections of maximum pixel intensity. Settings for laser power, pinhole and photo multiplier tube (PMT) were kept constant in cases of comparative studies.

4.2. Hair cell labelling

To follow hair cell death and regeneration, gentamicin-treated sensory epithelia were labelled with the specific hair cell markers HSC-1 (1:100) and HCA (1:200). HCS (a kind gift of Prof. J. Corwin, University of Virginia) labels the cytoplasms of hair cells in all vertebrate classes; HCA (a kind gift of Dr. G. Richardson, University of Sussex) is associated with the surfaces of the stereocilia of vertebrate hair cells.

4.3. Bromodeoxyuridine labelling

The fixed organotypic cultures, placed on multispot slides, were permeabilised for 1 hour in PBS containing 0.2% TX-100 followed by a 1 hour incubation in 2 N hydrochloric acid (Sigma) to denature the DNA. After 5 washes in PBS, the tissues were preblocked for 1 hour in 10% GS/0.2% TX-100/PBS. The wholemounts were then incubated for 2-3 hours in mouse anti-BrdU antibody (Dako) diluted 1:100 in blocking solution. After a thorough wash in PBS, the wholemounts were incubated in FITC-conjugated anti-mouse-IgG for 1 hour in the dark. The nuclei were counterstained with 5 μg/ml propidium iodide (PI; Molecular Probes) in blocking solution containing
100 µg/ml DNase free ribonuclease (Sigma) for 45 min at 37°C. After final washes in PBS, the wholemounts were mounted in antifade-medium and the coverslips, placed on nail-varnish supports, were sealed with nail varnish.

The wholemounts were examined on a confocal microscope using a 40x oil immersion objective with a numerical aperture of 1.25. FITC and propidium iodide (ex/em 535/617 nm) were excited with the 488 and 568 nm laser lines, respectively. Montages of Z-series of the entire wholemounts were taken manually or using a computer controlled stage by scanning the sensory epithelia in 3 µm sections.

4.4. Immunogold labelling

Immunogold labelling of thin sections of a basilar papilla at E21 was used to verify the localisation of Cx43 and Cx30-antibodies within gap junction plaques. The basilar papilla was fixed directly in 2% PFA in PBS (pH 7.6) for 45 min. All steps were carried out in processing baskets rotating at room temperature, unless otherwise stated. After a thorough wash in PBS, the tissue was dehydrated in graded alcohol (30%-50%-70%-95%-9x100%) for 10 min per step. The tissue was infiltrated with LR gold resin (Agar Scientific) diluted 1:1 in absolute ethanol, followed by 100% LR gold resin and 100% LR gold resin containing 0.2% benzoin methyl ether initiator (Sigma) for 1 hour each. The tissue was incubated overnight in fresh benzoin-resin. After a further resin change, the tissue was embedded in gelatine capsules (Agar Scientific), filled with benzoin-resin, and polymerised with UV light at -20°C for 16 hours on a freeze substitution platform (RMC). Ultrathin sections of the tissue were cut using glass knives (cut by Graham Nevill on Ultracut E, Reichert & Jung) and placed on nickel grids.

The sections were pre-blocked in droplets of PBS containing 0.15% Tween 20 and 10% GS for 1 hour at room temperature. The sections were incubated overnight in a moist atmosphere at 4°C in droplets of rabbit anti-Cx43 (Sigma) and mouse anti-Cx30 antibodies (Zymed) diluted 1: 100 in PBS containing 0.15% Tween and 100 mM 1-Lysine. Following 5 washes in PBS/Tween, the grids were incubated in anti-rabbit IgG conjugated to 5 nm gold particles and anti-mouse IgG conjugated to 15 nm gold particles, both diluted 1:30 in PBS/Tween/Lys for 1 hour at room temperature. The sections were washed several times in PBS, followed by distilled water. The sections were counterstained with urayl acid for 10 min, washed several times with distilled water and stained with lead citrate for 3 min in a NaOH-atmosphere to prevent precipitation of lead carbonate. The sections were examined in a transmission electron microscope (Jeol 1010).
5. Western blot analysis

Western blotting was used to assess the specificity of the antibodies to mammalian Cx43 (Sigma), Cx30 (Zymed), and Cx26 (Gap 28H) in the basilar papilla and utricular macula. HeLa cells transiently transfected with Cx26, Cx30 and cx31 (see II.6.2) and stably transfected Cx43-HeLa cells were used as positive controls.

Cochlear ducts and utricles were dissected in PBS as outlined above and frozen in liquid nitrogen until further use. Transiently transfected HeLa cells were grown to confluency in an 80 cm² flask (Nunc), harvested by trypsination and centrifuged at 1000 rpm to remove the culture medium. The samples (~0.1 mg/µl) were sonicated (UP100H, Dr. Hielscher GmbH) in SB20 buffer (see appendix), heated to 70°C for 3 min, and centrifuged at 1000 rpm to remove cell debris. The total protein concentration of the homogenates was determined using a commercially available kit (BCA Protein assay kit, Pierce) with bovine serum albumin (BSA) as the standard. The supernatants were then stored at -20°C until further processing.

The denatured proteins were separated according to their molecular size using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Fifty micrograms of protein were loaded along with a mixture of rainbow and biotinylated protein molecular weight markers (MW 10 – 250 kDa and 14.4 – 970 kDa; Amersham) on a 10% (Cx43) or 12.5% (Cx26, Cx30) polyacrylamide gel (see appendix) and run at 150 V for 1.5 hours on a vertical gel electrophoresis unit (Jencons). The protein bands were then electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon, Millipore) in transfer buffer (see appendix) for 14 hours at 20 V.

Following the wetting in methanol for 5 min, the membranes were incubated for 1 hour in PBS containing 5% skimmed milk powder and 0.1% Tween-20 (Sigma) to block non-specific binding sites on an orbital shaker at room temperature. The membranes were sealed in plastic sheets and incubated in primary antibodies diluted in the blocking solution for 1 hour. The optimal concentration of the primary antibodies was determined empirically as 1:5000 for C6129 (Sigma), 1:500 for MAB3067 (Chemicon), 1:500 for 33-2500 (Zymed), and 1:200 for Gap 28H (a kind gift from Prof. W.H. Evans, Cardiff). Before the incubation in the secondary antibodies, the membranes were washed in PBS/Tween for 30 min with 5 changes of wash buffer. The membranes were then incubated for 1 hour in PBS/Tween containing HRP-conjugated anti-rabbit IgG (1:7000, Chemicon) or anti-mouse IgG (1:7000, Chemicon), and HRP-conjugated streptavidin (1:10000, Chemicon) for the detection of the biotinylated protein standards. After 5 washes in PBS/Tween, the HRP-labelled antigens were
detected by chemiluminescence, based on the oxidation of luminol catalysed by HRP and hydrogen peroxide. The membranes were incubated at room temperature for 2 min in the freshly prepared detection agent (ECL western blotting reagent, Amersham). The excess solution was drained off and the membranes were placed in an X-ray film cassette (Amersham), covered by a clear film. In the dark room, the light emitting membrane was exposed to an autoradiography film (Hyperfilm ECL, Amersham) for 5-10 min. The film was developed and fixed using standard procedures, and the image digitised by scanning the film.

6. Expression of chicken Cx31

The recently identified chicken Cx31 (Heller et al., 1998) was exogenously expressed in HeLa cells in order to corroborate the immunodetection of this connexin-isoform with antibodies to mammalian Cx26 and Cx30. The cDNA of cCx31, inserted between the EcoRI and Xhol restriction sites of the pADGal-4 vector was a kind gift from Dr. Stefan Heller (Harvard Medical School, USA). Owing to the lack of a promoter recognised by eukaryotic RNA polymerases, the cCx31-DNA had to be subcloned into the expression vector pCR3 (Invitrogen), which contains the cytomegalovirus promoter and, for selection in prokaryotic cells and eukaryotic cells, genes conferring resistance to ampicillin and to neomycin/kanamycin, respectively.

6.1. Subcloning of chicken Cx31

First, the recombinant plasmid was taken up and expressed by *Escherichia coli* and the transformed bacteria selected by their resistance to ampicillin. 5 μl of the cCx31/pAD GAL-construct were added to 45 μl of ultracompetent *E.coli* (XL10-Gold, Stratgene) and incubated for 30 min on ice. One tube contained 1 μl pUC18 (Stratagene) as a positive control, another solely *E.coli* as the negative control. The plasmid/bacteria solution was heat shocked in a 42°C waterbath for 90 s followed by a 5 min-incubation on ice. 400 μl LB broth (Invitrogen) was then added to each tube and incubated, shaking at 250 rpm, for 1 hour at 37°C. Positive clones were selected by spreading 200 μl from each tube onto separate agar plates (LB agar, Invitrogen) containing 50 μg/ml ampicillin and incubated overnight at 37°C. The following day, 4 ml selective LB broth were inoculated with a single *E.coli* colony under semisterile conditions and incubated for 5 hours at 37°C. Each bacteria suspension was spilt into 4 tubes and grown to saturation at 37°C on a shaker. The next day the tubes were centrifuged for 10 min at 2000 rpm to remove the supernatant. The Qiagen plasmid mini
kit (Qiagen) was used to isolate the plasmid DNA. The purification is based on the binding of the plasmid DNA to an anion-exchange resin. Prior to the elution of the DNA in a high-salt buffer, RNA, proteins, and other impurities were removed by a medium salt-wash. Finally, the plasmid DNA was concentrated and desalted by isopropanol precipitation, and suspended in 30 μl TE buffer (Qiagen). cCx31 was then cut from the pAD-GAL vector by digesting the plasmid with the restriction endonucleases EcoRI and XhoI. 1.5 μl of EcoRI and XhoI (Promega) were added to 15 μl of the purified plasmid DNA, 4 μl enzyme buffer H (Promega), and 18 μl distilled water and incubated at 37°C for 30 min. Parallel to the digestion of the cCx31/pAD GAL-construct, a recombinant plasmid containing the pCR3 vector (Invitrogen) was cleaved with the same endonucleases to provide matching ligation sites and remove its insert. The DNA-inserts were then separated from their vectors by agarose gel electrophoresis. 30 μl DNA were loaded on a 1% agarose gel containing ethidium bromide (see appendix) along with 1 μl DNA standards (New England Biolabs) and run at 100 V for 30 min. The cCx31 and pCR3 bands were then cut from the gel, made visible by the nucleic acid stain ethidium bromide under UV light. The plasmids were extracted from the gel and purified using the Qiaquick gel extraction kit (Qiagen). To estimate the amount of recovered DNA the band intensity of the sample DNA was compared with that of known standards on an agarose gel. The samples with the highest yield of DNA were selected for ligation. A DNA ligase, which catalyses the formation of phosphodiester bonds between the ends of restriction fragments, was used to covalently join the cCx31-DNA and the pCR3-plasmid. To optimise the ligation process, samples with different vector to insert ratios (1:2, 1:4, 1:7) were prepared. 1 μl T4 DNA ligase (Promega), 1 μl ligase buffer (Promega) and distilled water was added to the different dilutions and incubated at room temperature for 2 hours.

The new constructs were introduced into ultracompetent E. coli (XL10-Gold, Stratagene) using heat-shock transformation as described above. Transformed cells were selected overnight on agar plates containing 50 μg/ml ampicillin at 37°C. Positive and negative controls along with a control for the internal plasmid ligation of the isolated pCR3 vector were carried out. The plasmid DNA of the transformed bacteria was purified using the Qiagen Plasmid Mini Kit, as outlined above. cCx31/pCR3 constructs were identified by analysing the EcoRI/XhoI cleavage products separated by agarose gel electrophoresis (Figure III.2A). 100 ml of LB broth were inoculated with a bacterial sample whose DNA insert had the same size as the original cCx31-DNA and the bacteria were grown to saturation overnight at 37°C on a shaker. Highly purified
cCx31-DNA was then isolated using a Quiagen plasmid maxi kit (Quiagen) and resuspended in 300 µl TE buffer. The DNA quantity and purity of the samples was estimated by measuring its extinction ratio at 260-280 nm on a spectrophotometer (UV1101, Biotech). The OD of ~1.8, indicated only a little protein contamination. The plasmid suspensions were diluted to a final concentration of 1 µg/µl and were stored at -20°C. A restriction digestion of the recombinant plasmid was used to characterise the cCx31-DNA insert (see Fig. III.2B). The following pairings of endonucleases were used for multiple digestion: Sal/Xhol, Xbal/EcoRI, Xbal/Xhol, PstI/Xhol, and EcoRI/Xhol (Promega). 1 µl of each enzyme was incubated with 3 µg cCx31-DNA in a buffer system, as recommended by the manufacturer, for 1 hour at 37°C. A sequential digest was used for SacI/Xhol, due to incompatible buffer conditions.

6.2. Transient and stable transfection of HeLa cells

Human HeLa cervix carcinoma cells (OHIO, ECACC) were transiently transfected with rat Cx26, mouse Cx30 (both DNAs inserted in pCR3, Marziano et al., 2003), and chicken Cx31. The recombinant plasmids were introduced into human HeLa cervix carcinoma cells (OHIO, ECACC) by endocytosis using the cationic transfection agent polyethylenimine. HeLa cell were grown under sterile conditions in tissue culture plate (35 mm) to 50-60% confluency in Dulbecco’s Modified Eagle medium (DMEM, Invitrogen) containing 10% foetal bovine serum (FBS), 1% penicillin/streptomycin, 0.5% fungizone and 2mM glutamine (all Sigma) at 37°C in a 5% CO2 atmosphere. To each dish 6 µl of jetPEI™ transfection reagent (Qbiogene) diluted in 100 µl of 150 mM NaCl were added to 3 µg DNA diluted in 100 µl of 150 mM NaCl, as recommended by the manufacturer. The mixture was immediately vortexed and spun down and incubated for 15-30 min at room temperature. 200 µl/well of the jetPEI™/DNA mixture was added to 1.5 ml of DMEM and incubated for 24-48 hours at 37°C.

For transient transfection, HeLa cells, which were directly grown on a coverslip placed inside the petridish, were co-transfected with 2 µg connexin-DNA and 1 µg pGreen lantern-GFP (Invitrogen) to visualise transfected cells under an inverted epifluorescence microscope (Nikon). After reaching ~50% confluencey, the HeLa cells attached to the coverslip were fixed in 4% PFA and processed for immunocytochemistry as described above.

To obtain stably transfected cCx31-HeLa clones, after the incubation with the jetPEI™/DNA mixture, the cells were rinsed with HBSS and harvested after a 5 min-incubation in 500 µl trypsin/EDTA solution (Invitrogen) at 37°C. The cell suspension
was split 1:5 to several culture dishes (10 cm diameter, Nunc) containing 10 ml DMEM and 500 μg/ml Geneticin (G418, Sigma). After seven days, by which time non-transfected and transiently transfected cells are thought to be dead, the concentration of G418 was reduced to 250 μg to prevent cytotoxic effects on HeLa cells. The selection medium was changed every 4 days for 2-3 weeks. Once cell clones reached a diameter of 2-3 mm, they were isolated using plastic cloning rings (Sigma) and harvested in 20 μl of selection medium after trypsination. The cells were grown to confluency in a 24-well tissue culture plate (Nunc), harvested and transferred to 35 mm dishes. After reaching confluency, HeLa cells with exogenous connexin expression were identified and characterised by immunocytochemistry. For that purpose, HeLa cells were directly grown on a coverslip until ~50% confluent. HeLa cells were fixed in 4% PFA and processed for immunocytochemistry as described above. The selection process for stably transfected cCx31-HeLa cells was still ongoing at the time of submission.

7. Fluorescence recovery after photobleaching

Intercellular coupling in the basilar papilla and utricular macula was examined in vitro using fluorescence recovery after photobleaching (FRAP). FRAP experiments were generally carried out on explants, which were cultured for one day. To study intercellular coupling after hair cell loss the explants were incubated in 1 mM gentamicin for another 24 hours.

The acteoxymethyl (AM) ester derivative of calcein was used to introduce a fluorescent tracer into the epithelia. Calcein AM is a nonfluorescent, uncharged and lipophilic molecule that permeates cell membranes. Inside live cells, the lipophilic blocking groups are cleaved by non-specific cytoplasmic esterases, releasing the fluorescent dye Calcein, which is well retained within cells. Calcein is a calcium-chelating agent and produces an intense green fluorescence (ex/em 495/515 nm) in the presence of bound calcium. The polyanionic (2+, 6-) fluorochrome with a molecular weight of 623 Da has been shown to pass through gap junctions (Goldberg et al., 1999).

The epithelia were loaded with 1-2 μM calcein AM (Molecular Probes), diluted in Medium 199 with Hanks' Salt solution from a 1 mM dimethyl sulfoxide (DMSO) stock solution. To keep extracellular hydrolysis of calcein AM to a minimum, serum was omitted from the loading medium. The majority of the tissues were incubated for 20-30 min at room temperature. In tissues with complete hair cell loss, the loading times had to be increased up to 2 hours, owing to the slow uptake of calcein AM into supporting cells. If the loading times exceeded 30 min, the tissues were kept at 37°C to
maintain the viability of the cells and accelerate the dye uptake. After loading, the cell culture dishes were washed several times with HBSS to remove the acteoxymethyl ester and prevent further loading during the measurements.

Measurements were taken within the next 2-3 hours on a confocal laser-scanning microscope (Leica TCS SP2). The tissues, attached to the membrane inserts, were maintained in Medium 199 with Hanks’ Salt with 25 mM HEPES, on a heating pad or in an incubator at 35±2 °C. The monitoring of cell viability was intrinsic to the experiment, with the use of calcein AM, a marker for live cells, as fluorescent tracer. Additionally, 3 μM propidium iodide (Molecular Probes), commonly used for identifying dead cells, was present in the medium.

A 63x water immersion objective lens with a numerical aperture of 0.9 (HCX APO L U-V-I 63.0x0.90 W) of an upright epifluorescence microscope (Leica) was used to focus just below the lumenal surface of the epithelium. Optical sections were scanned in xyt-mode, with an 8-bit resolution, and an Airy pinhole (178 μm width). A unidirectional scan at 400 Hz and zero averaging took 1 s 635 ms to scan a format of 512x512 pixels. The time-lapse-feature of the Leica confocal software (LCS) was used to automate the settings and allow the rapid switching between the pre-bleach, bleach and post-bleach sequences. An area of 119x119 μm (optical zoom 2) was recorded for the prebleach and postbleach scans at minimum laser power and the acousto-optic tuneable filter (AOTF) set at 20% for the 488 nm line. By increasing the optical zoom to 5, a 47.6 x 47.6 μm area was bleached at maximum laser power and 100% AOTF for 32 s, which resulted in an 80-95% bleach of fluorescence in that area. Excitation with a single photon laser line caused a double cone-shaped bleaching pattern, resulting in the loss of fluorescence throughout the depth of tissue (25-35 μm) (see Fig III.12). Fluorescence recovery was monitored for 5-10 min at intervals of 1 s 63 ms or 10 s.

To study fluorescence recovery within the depth of the epithelia the tissue was scanned at 1 μm sections using the xytz-mode. The scan format was reduced to 512x32 pixels to reach a scan speed between 5 and 7 s/frame, depending on depth of the tissue. Otherwise the same settings were used as in single-scan experiments. To bleach a section within this tissue-strip, the zoom was increased to 5 and the epithelium was scanned 10 times at maximum laser power. Fluorescence recovery was subsequently monitored for up to 50 frames at zoom 2 by the attenuated laser beam.
8. Appendix

**Western blot:**

**SB20 buffer**
- 20% Sodium dodecyl sulfate (SDS)
- 0.1 M Tris(hydroxymethyl)aminomethane (Tris)
- 10 mM Ethylenediaminetetraacetic acid (EDTA)

**Laemmli sample buffer**
- 2% SDS
- 60 mM Tris
- 0.02% bromophenol blue
- 10% glycerol
- 100 mM dithiothreitol (DTT)

**Laemmli running buffer (pH 8.3)**
- 0.04% SDS
- 20% Methanol
- 48 mM Tris
- 39 mM Glycine

**SDS-Page stacking gel (4ml)**
- 2.7 ml H₂O
- 0.67 ml 30% Acrylamide mix
- 0.5 ml 1 M Tris (pH 6.8)
- 0.04 ml 10% SDS
- 0.04 ml 10% APS
- 0.004 ml TEMED

**SDS-Page separating gel (10ml)**
- 10% / 12.5%
- 4 / 3.1 ml H₂O
- 3.3 / 4.2 ml 30% Acrylamide mix
- 2.5 ml 1.5 mM Tris (pH 8.8)
- 0.1 ml 10% SDS
- 0.1 ml 10% Ammonium persulfate (APS)
- 0.005 ml Tetramethylethylenediamine (TEMED)

**Agarose gel electrophoresis:**

**Agarose gel**
- 1% agarose in Tris acetate-EDTA buffer
- 0.5 µg/ml ethidium bromide

**Loading buffer**
- 6.5% sucrose
- 1 mM Tris
- 1 mM EDTA
- 0.02% bromophenol blue
III. GAP JUNCTIONS IN THE AVIAN INNER EAR

1. Introduction: Functional Morphology of the Avian Inner Ear

The Basilar Papilla

The cochlear duct contains the hearing organ, known as the basilar papilla and the macula lagena, a sensory epithelium with yet unknown function but similar morphology and physiology to the otoconial organs of the vestibular system. The basilar papilla and the lagena line the endolymphatic space of the scala media, which is separated from the perilymph of the scala tympani and scala vestibuli by the tegmentum vasculosum and basilar membrane, respectively (Fig. III.1A).

The basilar papilla, which ranges from 2 mm in the canary to 11 mm in the barn owl, is generally shorter than the organ of Corti of therian mammals and is not coiled but slightly curved and twisted. The basilar membrane, which is framed by the inferior and superior fibrocartilaginous plate (abneural and neural limbus), is tapered towards its proximal end, which gives the epithelium a sickle-shaped appearance. The cochlear ganglion, which defines the neural (superior) and abneural (inferior) edge of the basilar papilla, contains the somata of the afferent neurons of the cochlear and lagenar branch of the VIIIth nerve. Parallel with the widening of the basilar papilla, the number of hair
cells across the epithelium increases from 6-10 at the proximal end to 45-50 at the distal end. Depending on the length of the papilla, the total number of hair cells ranges from about 3,000 in the canary to over 15,000 in the barn owl (reviewed by Manley, 1990; Gleich and Manley, 2000; Necker, 2000).

Typically for an amniote hearing organ, the basilar papilla is tonotopically organised, that is the frequencies are mapped monotonically along the basilar papilla, with the proximal end (base) representing the highest and the distal end (apex) the lowest frequencies within the hearing range. Each hair cell responds to a characteristic frequency, which results in the highly tuned responses of the afferent nerve fibres. In mammals, frequency analysis depends largely on the sharp tuning and amplification of basilar membrane motion, which is achieved by the graded mechanical properties of the basilar membrane and the active movement of the outer hair cells (reviewed by Robles and Ruggero, 2001). Although the avian basilar membrane possesses some mechanical tuning (von Békésy, 1960; Gummer et al., 1987), frequency analysis in birds depends mainly on the electrical and micromechanical properties of the hair cells, which are determined by molecular and morphological gradients along and across the basilar papilla (reviewed by Gleich and Manley, 2000; Koeppel et al., 2000).

The membrane potential of avian hair cells undergoes dampened sinusoidal oscillations at a specific frequency in response to the depolarisation of the hair cell; so electrically tuning the hair cells. As with turtles and amphibians, the key elements of electrical tuning are voltage-activated Ca\(^{2+}\) channels and Ca\(^{2+}\)-activated K\(^+\) channels (reviewed by Fettiplace and Fuchs, 1999). These two currents produce an alternation of depolarisation and repolarisation of the hair cell membrane that results in electrical resonance. The resonant frequency, which represents the characteristic frequency of the hair cell, is correlated with the number and, more importantly, with the faster gating kinetics of Ca\(^{2+}\)-activated K\(^+\) channels. Thus, hair cells tuned to higher frequencies possess a greater number of K\(^+\) channels, which are also faster gating, than cells with a lower characteristic frequency.

Morphological gradients are evident in many parameters, for example the size and shape of hair cells, the characteristics of stereociliary bundles, the mass of the tectorial membrane, and number and size of afferent and efferent nerve terminals (reviewed by Manley, 1990; Gleich and Manley, 2000). Although there is no strict separation of inner and outer hair cells in the hearing organ of birds (rather there is a continual gradient in hair cell shapes along and across the basilar papilla) a division of labour within the hair cell population has been proposed. On the basis of both their position across the
epithelium and their innervation patterns, tall hair cells (THC) and short hair cells (SHC) have been compared to the mammalian inner and outer hair cells, respectively. Short hair cells and tall hair cells represent the extremes of the morphological differences among avian hair cells (Takasaka and Smith, 1971). Tall hair cells (THC), which resemble the typical hair cell of more primitive vertebrates, are tall and columnar in shape, and are primarily located at the distal end and along the neural limbus (Fig. III.1B). Short hair cells (SHC) are wider than they are tall and have larger cuticular plates (Fig. III.1C). They comprise up to 35% of all hair cells and are found proximally and on the abneural edge, overlying the free moving basilar membrane. THCs are predominately innervated by afferent fibres of the VIIIth nerve, which transmit auditory information from the sensory epithelium to the auditory cortex. In contrast, SHCs are mainly under the control of the efferent system, whose neurons in the brain stem send descending axons to the sensory epithelium.

Determined by the cells' response frequency, there is not only a gradient in hair cell shapes and innervation, but also a change in hair bundles along and across the epithelium. As with mammals, the hair bundle of birds consists of modified microvilli, which are hexagonally arranged into rows with increasing height at the apical surface of the hair cell. Each stereocilium is composed of a dense core of actin microfilaments with cross-linking proteins and anchored in the cuticular plate (Tilney et al., 1983). Depending on the species and animal's age, a kinocilium may be present behind the tallest row. The number of stereocilia per bundle increases from ~50 at the distal end to ~300 at the proximal end, which is paralleled by an increase in the number of transduction channels. The length and width of the stereocilia are also related to the cells' response frequency and vary significantly between species, depending on their hearing range. Generally, the height of the longest stereocilia increases proximally to distally, for example, from 1.5 μm to 5.5 μm in the chicken. The diameter of the stereocilia varies both across and along the papilla, reaching up to 0.19 μm in SHCs (Tilney and Saunders, 1983).

Birds are unique among tetrapods in that their hearing organ shows a systematic change in the orientation of their hair bundles. Hair cells located along the neural and abneural limbus have their bundles oriented nearly parallel to the edges of the papilla with the tallest row facing the abneural edge. However, cells in the centre of the epithelium, especially in the distal half, can have their bundle rotated up to 90°. The position of the rotated hair cells along the papilla varies with species and appears to be correlated with the hearing range (Fischer et al., 1988; Cotanche and Corwin, 1991).
In this highly organised epithelium, hair and supporting cells are arranged in a hexagonal mosaic, with each hair cell surrounded by six or seven supporting cells (Goodyear and Richardson, 1997). The somata of the non-sensory supporting cells (SC) extend from the basilar membrane to the endolymphatic lumen, into which the SC microvilli protrude. Within the sensory epithelia, two types of supporting cells - the epithelial supporting cells and the border cells on the abneural edge of the papilla - have so far been identified on morphological grounds (Oesterle et al., 1992).

A monolayer of specialised cuboidal epithelial cells, the hyaline cells, lines the abneural edge of the basilar papilla. Hyaline cells, which are also found in caimans (Drenckhahn et al., 1991), have no analogue in mammals. These cells, which are tightly attached to the basilar membrane, express contractile proteins (Cotanche et al., 1992; Oesterle et al., 1992) and receive efferent innervation (Takasaka and Smith, 1971; Oesterle et al., 1992). These features lead to the suggestion that hyaline cells actively regulate the mechanical properties of the basilar membrane, thereby modulating the passive mechanical tuning of the basilar membrane (Drenckhahn et al., 1991; Cotanche et al., 1992). In addition, they may contribute to the repair of a severely damaged papilla (Cotanche et al., 1995). Above the abneural limbus lie the unspecialised vacuole cells, on which the cuboidal cells border. At the neural edge, clear cells are located adjacent to the epithelial supporting cells. The actin-rich homogene cells, to which the tectorial membrane is anchored, form part of the neural wall of the cochlear duct.

The tectorial membrane (TM), responsible for the displacement of the hair bundles, is a thick (up to 110 μm at the neural edge), wedge-shaped extracellular matrix covering the entire papilla (Jahnke et al., 1969; Tanaka and Smith, 1975) and to which the stereociliary bundles of the hair cells and the microvilli of the supporting cells are firmly attached. The avian TM is composed of mainly two non-collagenous glycoproteins, α- and β-tectorin, which are synthesised by clear cells and supporting cells (Goodyear et al., 1996; Coutinho et al., 1999; Goodyear and Richardson, 2002).

The tegmentum vasculosum is, like its mammalian homologue, the stria vascularis, responsible for maintaining the ionic composition of the scala media. It is a highly folded, richly vascular epithelium, which forms the lateral and dorsal walls of the avian cochlear duct (Cotanche and Sulik, 1982; Hossler et al., 2002). The endolymphatic surface of the tegmentum vasculosum consists of a mosaic of alternating dark and light cells. The dark cells, equivalent to the marginal cells in the stria vascularis, are characterised by their electron dense cytoplasm and Na⁺, K⁺-ATPase activity (Schneider et al., 1987; Hara et al., 2002; Hossler et al., 2002). The light cells, which may be the
equivalent of intermediate and/or basal cells in the stria vascularis, are coupled by gap
junctions and are in contact with the capillary epithelium by which the fluids, salts and
metabolites for the active ion transport are provided (Hossler et al., 2002).

The endocochlear potential in the avian cochlea is, at around +20 mV (Necker,
1970), considerably smaller than the EP in the mammalian cochlea. This large
difference in the EP between mammals and birds, despite similar K⁺ concentrations
(140-160 mM) in the endolymph (Ninoyu et al., 1987; Runhaar et al., 1991; Sauer et al.,
1999), points to a modified generation of the avian EP, which may be associated with
the absence of an equivalent to the intrastral space in the tegmentum vasculosum.

The Utricle

The utricle is part of the otoconial end organs of the vestibular system that are
responsible for the detection of linear acceleration and the position of the head relative
to the gravitational field. In its mature form, the flat and rhomboid-shaped utricular
macula measures 2 mm² and contains about 37,000 hair cells, each of which is
surrounded by 4-5 supporting cells (Goodyear et al., 1999). Transitional cells separate
the sensory epithelium from the ion-transporting epithelium, which is composed of a
monolayer of dark cells. The hair cells are covered by the otoconial membrane, an
extracellular matrix on top of which calcium carbonate crystals, the otoconia, are
embedded. The sensory epithelium is divided into extrastriolar and striolar regions, the
latter being characterised by the reversal of the hair bundle orientation and a thinning of
the otoconial layer.

Based on morphology, innervation, and channel properties, two types of hair cells
are distinguished in the utricular macula. The Type I hair cells are pear shaped and
enclosed by an afferent calyx. Type I hair cells are confined to the striola and have
distinctive slow outward potassium currents (Correia and Lang, 1990; Weng and
Correia, 1999)). Following the membrane depolarisation of Type I hair cells, a
shortening of the hair cell of up to 4% has been demonstrated, which may cause the
tilting of the cuticular plate and the modification of the stiffness of the stereocilia (Ogata
and Sekitani, 1993). Type II hair cells have an elongated cylindrical appearance and
their basal surfaces make contact with several bouton-type nerve endings. They exhibit
fast outward potassium currents and are predominantly located in the extrastriolar
region (Correia and Lang, 1990; Weng and Correia, 1999). Unique to birds, Type II hair
cells also line the morphological reversal line in the centre of the striola (Jørgensen and
Anderson 1973; Rosenhall 1970). The differential distribution of the two receptor types
and their distinct ionic conductances may lead to different filter properties in the striolar and extrastriolar regions (Weng and Correia, 1999). In addition, vestibular hair cells show a distinct orientation pattern of their bundles, underlining the directional sensitivity of each vestibular hair cell. Across the reversal line in the striola of the utricular macula, the hair bundles on both sides are oriented with the kinocilium facing the striola (Rosenhall, 1970). Thus, hair cells on opposite sides of the striola have opposing morphological polarisations such that the shearing motions of the otoconial membrane excite one hair cell group while inhibiting the other.

2. Results: Gap Junctions in the Avian Inner Ear

2.1. Connexin Expression in the Avian Inner Ear

The composition and distribution of gap junctions within the basilar papilla and the utricular macula of chick hatchlings was examined by immunohistochemistry using antibodies to the mammalian Cx26, Cx30 Cx43, and to the chicken Cx31, as described in chapter II.

2.1.1. Data analysis

To quantify the number and size of immunodetectable gap junctions within supporting cells, wholemount preparations of basilar papilla and utricular macula were labelled with a monoclonal antibody to mCx30 (33-2500, Zymed) and examined by confocal laser microscopy. The proximal and distal ends of the basilar papilla, and the extrastriolar region of the utricular macula were scanned at 1 µm intervals with a 512x512 resolution using a 63x objective, which resulted in a pixel size of 0.47 µm². Projections of 3–4 optical sections within the lumenal and basal regions of supporting cells were generated using the LCS lite software package (Leica) and subsequently imported as greyscale images into ImageJ 1.31 (NIH). The pixel intensity thresholds were interactively set for the labelled gap junctions and converted into binary images. The number, the average and total areas of particles larger than 2 pixels were automatically measured within an area of 100 µm².

Differences between the lumenal and basal values within supporting cells were statistically analysed using a paired Student’s t-test, while the data of the various regions within the epithelia were compared using a one-way ANOVA with the Bonferroni post test (GraphPad Instat).
2.1.2. Characterisation of the cCx31 plasmid

The cCx31-DNA was subcloned into the expression vector pCR3 (Invitrogen) as described in chapter II. cCx31/PcR3 constructs were identified from their cleavage products following the digestion with the endonucleases EcoRI and Xhol, whose restriction sites flank the cCx31-DNA insert (Fig. III.2A). To ensure the completeness of the cCx31-DNA coding region, a restriction map of the DNA insert was obtained by multiple digestion with SacI, XbaI, PstI, EcoRI, and Xhol (Fig. II.2B). SacI and XbaI were predicted to cut one site at the beginning (211) and towards the end (860) of the coding sequence (196-987), respectively. PstI recognised three restriction sites within the coding region at the base pairs 216, 316 and 435 (Fig. III.2C). Analysis of the products of the different cleavage reactions separated by gel electrophoresis revealed a complete coding region within the 2 kbp insert. Approximately 100 missing base pairs at the 3' untranslated region (UTR) and about 800 additional base pairs at the 5'UTR account for the differences in molecular weights of the published cDNA (1328 bp, Heller et al., 1998) and the cDNA supplied by Dr. Heller.

![Image of gel electrophoresis](image)

Figure III.2. Identification and characterisation of cCx31/pCR3 constructs.

(A) Cleavage products of the original pad Gal/cCx31 and pCR3/Cx26 constructs and the newly generated pCR3/cCx31 plasmid. (B) Restriction map of the cCx31/pCR3 construct. EcoRI and Xhol cut at the beginning and the end of the cCx31-insert. SacI, XbaI and PstI sites are localised within the coding region. The fragment in the EcoRI/XbaI digestion marked by an asterisk is due to a further XbaI site within the pCR3 vector. (C) Restriction sites recognised by PstI, SacI and XbaI within the coding region (arrow) of cCx31.
2.1.3. Characterisation of antibodies

The examination of connexin expression was focused on cCx31, Cx43, Cx26 and Cx30 as the mRNAs of cCx31 and Cx43 have already been identified in the inner ear of the chicken (Heller et al., 1998; Coutinho et al., 2000), and Cx26 and Cx30 constitute the main connexin isoforms in the mammalian inner ear (Lautermann et al., 1998; Forge et al., 2003a).

Attempts were made to raise a polyclonal antibody against a synthetic peptide with a cCx31-specific sequence (amino acids 215-229, TKFFLRRSRKAGNQK) using a commercial service for the antibody production (Sigma Genosys). However, the supplied antiserum failed to provide any immunoreactivity in both Western blot and immunohistochemistry. Consequently, the high sequence similarities of cCx31 with the cytoplasmic loop of Cx26 and the C-terminal of Cx30 (Fig. III.3) were utilised to detect cCx31 using the Cx26 antibody Gap 28H and the Cx30 antibody 33-2500 (Zymed). The amino acid sequence in the cytoplasmic loop of rat Cx26, against which Gap 28H was raised, is 75% identical with the respective cCx31-sequence. The immunogen for the antibody 33-2500 was derived from the last 22 amino acids of the C-terminus of mouse Cx30, which shares a 73% homology with cCx31.

| cCx31  | 1        | MDNGALOTILGGVNKHSTSIGKIWLTVLPFIRIMILVVAEEVGDEQDFCNTLQPGC |
| mCx30  | 1        | MDNGALOTILGGVNKHSTSIGKIWLTVLPFIRIMILVVAEEVGDEQDFCNTLQPGC |
| mCx26  | 1        | MDNGALOTILGGVNKHSTSIGKIWLTVLPFIRIMILVVAEEVGDEQDFCNTLQPGC |
| cCx31  | 61      | KNYCDHFIPFSHIRMALQLOIFVSTPALLVAMHVAYRHHEKKKFKLGKIDIDEE |
| mCx30  | 61      | KNYCDHFIPFSHIRMALQLOIFVSTPALLVAMHVAYRHHEKKKFKLGKIDIDEE |
| mCx26  | 61      | KNYCDHFIPFSHIRMALQLOIFVSTPALLVAMHVAYRHHEKKKFKLGKIDIDEE |
| cCx31  | 121     | HITCKVRIEGSLMWTTSIFSRPEAVFMYPFYPMYGFIPXPRMCKAWPCTPVVLK |
| mCx30  | 121     | HITCKVRIEGSLMWTTSIFSRPEAVFMYPFYPMYGFIPXPRMCKAWPCTPVVLK |
| mCx26  | 121     | HITCKVRIEGSLMWTTSIFSRPEAVFMYPFYPMYGFIPXPRMCKAWPCTPVVLK |
| cCx31  | 181     | FISRPTEKTVPTFMILVSICILLNVAVITYLLKRSFLRSSAGAGNHSMHNLKET |
| mCx30  | 181     | FISRPTEKTVPTFMILVSICILLNVAVITYLLKRSFLRSSAGAGNHSMHNLKET |
| mCx26  | 181     | FISRPTEKTVPTFMILVSICILLNVAVITYLLKRSFLRSSAGAGNHSMHNLKET |
| cCx31  | 241     | KONEMNELISDSQNTWIGPS |
| mCx30  | 240     | KONEMNELISDSQNMFPSE |
| mCx26  | 240     | KONEMNELISDSQNMFPSE |

Figure III.3. Alignment of the amino acid sequences of the avian connexin 31 (cCx31) with the murine connexin 26 (mCx26) and connexin 30 (mCx30).

The alignment of the connexin sequences using the BLAST algorithm revealed that the N-terminus, the transmembrane domains, and the extracellular and cytoplasmic loops of cCx31 align better with mCx26 (84% identical), whereas the C-terminus is closer to mCx30 (72% identical). The sequences of the immunogenic peptides for the Cx26 and Cx30 antibodies appear in italic script.
Figure III.4. Exogenous expression of Cx26, Cx30 and cCx31 in HeLa cells. Labelling of cCx31-HeLa cells, co-transfected with GFP (green), with the polyclonal Cx26 antibodies Gap 28H (A') and 33-5800 (B'), and the monoclonal Cx30 antibody 33-2500 (C') resulted in punctate staining (red) between apposing cells, except for (B'). This confirms the cross-reactivity of Gap 28H and 33-2500, but not 33-5800, with cCx31. Cx26-HeLa cells (A,B) and Cx30-HeLa cells (C) were used as positive controls for the respective antibodies. Bar = 15 μm.
This predicted cross-reactivity of Gap 28H and 33-2500 with cCx31 was corroborated immunocytochemically using HeLa cells transiently transfected with cCx31-cDNA (Fig. III.4.). Incubation of the HeLa-cCx31 transfectants with Gap 28H and 33-2500 resulted in a punctate staining of membranes between apposing cells, which is characteristic for gap junctions (Fig. III.4A,B). This immunofluorescence was regarded as connexin-specific, as no immunoreactivity was observed in wild type HeLa cells (data not shown). No gap junction-specific signal was detected in cCx31-HeLa cells incubated with 33-5800 (Zymed; Fig.III.4C), a Cx26 antibody raised against a sequence in the cytoplasmic loop that provided positive staining in HeLa-Cx26 transfectants. Western blot analysis of homogenates of whole cochlear ducts and utricles of chick hatchlings confirmed the reactivity of both Gap 28H and 33-2500 with a protein having a molecular weight of 31 kDa (Fig. III.5A,B). No bands with a migration profile between 26 and 30 kDa were detected for either antibody. Homogenates of HeLa cells transiently transfected with cCx31, mCx30, and rCx26 were chosen as positive controls for the respective connexin isoforms. However, the probing with Gap 28H and 33-2500 failed to detect specific protein bands, possibly due to connexin yields below the sensitivity of the detection system. From the analyses of their migration profiles in Western blots and their gap junction-specific immunoreactivity in cCx31-HeLa cells, it was concluded that Gap28H and 33-2500 provided in the absence of other orthologues “cCx31-like” immunolabelling in the avian inner ear.

Figure III.5. Western blot analyses of antibodies to Cx26, Cx30 and Cx43.

Homogenates of cochlear ducts and utricles were resolved on a 12.5% (A, B) and 10% (C, D) acrylamide gel and probed with the polyclonal Cx26 antibody Gap 28H (A), the monoclonal Cx30 antibody 33-2500 (B), the polyclonal Cx43 antibody C6129 (C), and the monoclonal Cx43 antibody MAB3067 (D). Homogenates of HeLa cells stably transfected with mCx43 served as positive control for Cx43-migration. The detection of slow migrating bands (~ 70 kDa) is due to an unspecific reaction of the secondary antibody.
An orthologue with 92% of its amino acids identical to the mammalian Cx43 has been identified in the chicken (Musil et al., 1990). Despite this high sequence similarity, reliable and robust staining in the avian inner ear could only be obtained with the polyclonal antibody C6129 (Sigma), which is raised against the last 21 amino acids of the C-terminus of murine Cx43 and has a 94% homology with the respective avian sequence. Western blot analysis of homogenates of inner ear tissue confirmed the specificity of C6129 for cCx43 (Fig. III.5C). C6129 recognised three bands between 42 and 44 kDa in both the cochlear duct and utricle. The comparison of the migration pattern with that of chicken lens and pineal gland homogenates (Musil et al., 1990; Berthoud et al., 2000) revealed that the two main bands migrated similarly as the phosphorylated forms P1 (~ 43 kDa) and P2 (~ 44 kDa). In contrast to the homogenate of Cx43-HeLa cells, the non-phosphorylated form of Cx43 (~ 42 kDa) was only weakly expressed in the inner ear of chick hatchlings. Consistent with immunohistochemistry, no bands where detected in the chicken inner ear using the monoclonal antibody MAB3067 (Chemicon), which was raised against a peptide sequence derived from the cytoplasmic domain of mouse Cx43 (Fig. III.5D).

2.1.4. Connexin expression

Basilar papilla. The double labelling of transverse sections of the cochlear duct with the polyclonal Cx26 antibody Gap 28H and the monoclonal Cx30 antibody 33-2500 resulted in extensive immunofluorescence throughout the cochlear duct (Fig. III.6A). Immunoreactive punctae were visible in supporting cells (Fig. III.6B), homogene cells, clear cells, hyaline cells and cuboidal cells, and in the tegmentum vasculosum. Gap28H and 33-2500 co-localised in virtually all labelled gap junction plaques of the cochlear duct. No immunostaining was detected in the cochlear duct using the Cx26 antibody 71-0500 (data not shown). As shown in surface views of the basilar papilla (Fig. III.6C), linear gap junctions outlined the cell bodies of hyaline, cuboidal, clear and homogene cells, indicating that neighbouring cells were connected both longitudinally and laterally. Optical sections close to the basilar membrane revealed a uniform cCx31-like immunostaining between the cells, suggesting the epithelial cells of the cochlear duct are connected via gap junctions at their basal ends to form a continuous syncytium.

Figure III.7. Gap junction distribution in the auditory and vestibular sensory epithelium. Contrasting projection views of cCx31-like gap junction plaques in the luminal (middle) and basal (right) ends of supporting cells of (A) the distal and (B) the proximal regions of the basilar papilla, and (C) the extrastriolar region of the utricular macula. The left panel shows the respective Z-projections of phalloidin-labelled hair cells. Bar = 10 μm.
Figure III.6. Cx31 expression in the basilar papilla. Double labelling with a polyclonal antibody to Cx26 (Gap 28H; Alexa Fluor 633) and a monoclonal antibody to Cx30 (33-2500; FITC) resulted in turquoise immunofluorescence throughout the cochlear duct due to the overlapping staining patterns of the antibodies. TRITC-conjugated phalloidin was used as a counterstain for the F-actin-rich hair bundles and homogene cells. (A) Low power view of a cryosection of the distal cochlear duct, showing immunostaining in supporting cells (SC), hyaline and cubodial cells (HyC, CuC), in the tegmentum vasculoum (TV), and in homogene and clear cells (HoC, CC). Bar = 40 μm. (B) Higher magnification of the basilar papilla. Asterisks indicate tall hair cells (THC) and short hair cells (SHC), outlined by gap junctions of the surrounding supporting cells. Bar = 20 μm. (C) Surface view of the distal region of the basilar papilla. Bar = 20 μm.
Figure III.7. Gap junction distribution in the auditory and vestibular sensory epithelium.
cCx31-like gap junction plaques within supporting cells at both the distal and the proximal end of the basilar papilla showed a distinct distribution pattern, with relatively small punctate plaques at the lumenal surface and large sheet-like plaques at the basal surface near the basilar membrane (Fig. III.7A,B). This observation was confirmed by quantification of the total plaque area (Fig. III.8C), which was significantly different between the lumenal and the basal regions of supporting cells (distal: p<0.05; proximal: p<0.01). At the distal region of the basilar papilla this difference is due to an increase in both plaque numbers (p<0.05) and average plaque area (p<0.05), whereas at the proximal region this is due solely to an increase in the plaque area (p<0.01). From this it can be seen that the average basal gap junction plaques at the proximal end of the basilar papilla are significantly (p<0.01) larger than those at the distal end. However, measurement of lumenal plaques showed little difference in either average area or numbers between the distal and the proximal regions of the basilar papilla (Fig. III.8A,B).

Figure III.8. Quantitative analysis gap junction plaques at the lumenal and basal ends of supporting cells.
The means (±SD; n=5) of (A) the number, (B) the average area, and (C) the total area of cCx31-positive gap junction plaques in the distal (BPdist) and proximal (BPprox) regions of the basilar papilla and the extrastriolar utricular macula (UT). (+) indicates significant differences between the various epithelial regions (one-way ANOVA); (*) indicates significant differences within supporting cells of a given region (paired Student’s t-test; p<0.05).
Figure III.9. Cx43 expression in the basilar papilla. Double labelling of cryosections of the basilar papilla with a polyclonal antibody to Cx43 (C6129; Alexa Fluor 633) and a monoclonal antibody to Cx30 (33-2500; FITC). F-actin was detected by TRITC-conjugated phalloidin. (A) Low power view of the cochlear duct. Immunofluorescence for Cx43 is confined to supporting cells (SC) of the basilar papilla and to the abneural and neural limbus (aL, nL). Bar = 40 µm. (B) Higher magnification of transverse sections of the distal (top), middle (mid) and proximal (bottom) regions of the basilar papilla. Turquoise immunofluorescence is due to the co-localisation of Cx43 and eCx31-like antibodies within gap junction plaques. Arrowheads indicate the concentration of Cx43-positive gap junctions beneath the basal surfaces of tall hair cells overlying the neural limbus. Bar = 20 µm.
Figure III.10. Co-localisation of Cx43 and cCx31 in gap junction plaques.
In contrast to the ubiquitous cCx31-like labelling, Cx43-immunoreactivity was confined to the supporting cells of the basilar papilla and the neural and abneural limbus (Fig. III.9A). Within the basilar papilla, the size and the density of Cx43-positive gap junction plaques gradually increased from distal to proximal (Fig. III.9B & 15B’,C’). At the distal end, Cx43-labelling showed a relatively homogenous distribution across the sensory epithelium, with the exception of supporting cells along the neural and abneural edges where only isolated Cx43-positive plaques were detected. However, towards the proximal end of the basilar papilla, Cx43-immunoreactive sites were concentrated below the basal surfaces of tall hair cells overlying the neural limbus. Cx43 and cCx31-like immunostaining overlapped extensively, thus pointing to a co-assembly of Cx43 and cCx31 within gap junction plaques. Confocal images taken at higher magnification revealed that Cx43-immunofluorescence was either segregated into discrete punctae within cCx31-like plaques or was concentrated on one side of the plaque (Fig. III.10A).

Immunogold labelling of ultrathin sections of the basilar papilla confirmed the co-localisation of Cx43 and cCx31 within gap junction plaques. In the preliminary analysis of immunoreactive gap junctions, gold markers of both isoforms were found on either sides of the junction profiles. (Fig. III.10B,B’).

**Utricular macula.** Double labelling of cryosections of the utricular macula with Gap 28H and 33-2500 resulted in extensive and overlapping immunostaining among the populations of supporting cells and transitional cells (Fig. III.11A,B). By contrast, only isolated plaques were immunoreactive in the dark cell region (Fig. III.11A).

The cCx31-like immunostaining within supporting cells was concentrated at their lumenal and basal ends. The immunoreactive sites were linear and closely mirrored the hexagonal shape of supporting cell somata, especially near the basement membrane (Fig. III.7C). The area of cCx31-like plaques at the lumenal and the basal ends of supporting cells was not significantly different, but there were significantly (p<0.05) more gap junction plaques present in the basal region of supporting cells (Fig. III.8A). The number of both basal and lumenal plaques was significantly higher than in the basilar papilla. This led to a significantly higher total plaque area (distal: p<0.001; proximal p<0.01) at the lumenal surface of supporting cells in the utricular macula.

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Figure III.10. Co-localisation of Cx43 and cCx31 in gap junction plaques. (A) Surface views of the lumenal and basal regions of supporting cells in the basilar papilla. Double labelling for Cx43 (blue) and cCx31 (green) resulted in the overlap of immunofluorescence. Arrows indicate possible heterotypic junctions; arrowheads mark segregated Cx43 domains within cCx31-like gap junctions. Bar = 5 μm (B) Double immunogold labelling of Cx43 (5 nm gold, arrows) and mCx30 (15 nm gold) in an ultrathin section of the basilar papilla. Both sizes of gold particles are found on both sides of the junction profile. Bar = 100 nm
Figure III.11. Connexin expression in the utricular macula. Double labelling of the utricular macula with a monoclonal antibody to Cx30 (33-2500; FITC) and a polyclonal antibody to Cx43 (C6129; Alexa-Fluor 633) or Cx26 (Gap 28H; Alexa Fluor 633). F-actin was detected by TRITC-conjugated phalloidin. (A) Low power view of a cryosection of the utricle. CcX31-like immunostaining was present in the supporting cells (SC) of the utricular macula and the lateral crista (LC), and the cells of the transitional zone (TC), but not in the dark cells (DC). Cx43-positive gap junctions were confined to the stroma (St). Bar = 40 μm. (B&C) Higher magnification of the utricular macula. Bar = 20 μm. (D) Projection view of a wholemount utricular macula and (D′) a corresponding optical section within the lumenal region of the epithelium. The asterisk marks a hair cell surrounded by supporting cells with diffuse cytoplasmic Cx43-immunostaining. Bar = 15 μm.
compared with those in the basilar papilla (Fig. III.8C). While the average area of basal plaques in the utricular macula was smaller (proximal: p<0.05) than those of the basilar papilla (Fig. III.8B), their much larger numbers resulted in a similar total area compared with the distal end of the basilar papilla, although there still remained a significant difference (p<0.001) when compared with the proximal end of the basilar papilla.

Punctate immunoreactive sites for Cx43 were detected in the connective tissue underlying the sensory epithelium but virtually no labelling was detected at membrane sites between supporting cells (Fig. III.11A,C). Instead, diffuse staining in the cytoplasm of supporting cells was observed (Fig. III.11D).

2.2. Intercellular Coupling in the Basilar Papilla and Utricular Macula

Gap junctional communication between supporting cells was examined by fluorescence recovery after photobleaching (FRAP) adapted to visualise dye spread in the intact inner ear tissue.

2.2.1. Fluorescence recovery after photobleaching (FRAP)

The FRAP technique is based on the irreversible photobleaching of fluorescent molecules by a high powered laser beam and the subsequent recovery of fluorescence as surrounding non-bleached fluorescent molecules diffuse into the bleached area (reviewed by Lippincott-Schwartz et al., 2001; Reits and Neefjes, 2001). FRAP enables the determination of the mobile fraction of the fluorescent molecule and its diffusion constant. The mobile fraction provides a measure of the extent to which the molecules can move within cells and is determined by the ratio of the final to the initial fluorescence intensity in the bleached region. The diffusion constants provide a measure of the rate of the molecules’ movements and are obtained by plotting the recovery of the relative fluorescence intensity within the bleached region as a function of time, and fitting this recovery curve with various equations.

The FRAP technique was originally developed in the 1970s to visualise the lateral movement of fluorescently tagged proteins in cell membranes (Axelrod et al., 1976). A decade later, FRAP was adapted to measure gap junction-mediated exchange of fluorescent tracers between cells (Wade et al., 1986). In the “gap-FRAP” method, a membrane- and gap junction-permeant fluorescent tracer is introduced at uniform concentrations into cells and a concentration gradient is created by photobleaching the fluorochrome in selected cells. If the bleached cells are connected via gap junctions with adjacent non-bleached cells, the tracer diffuses into the bleached cells and the
fluorescence recovers. The FRAP method is a widely used non-invasive way to qualitatively and quantitatively examine the permeability of gap junctions for fluorescent tracers in a tissue or cell monolayer, and to determine the effects of various gap-junction blockers and regulators on intercellular coupling (e.g. Pluciennik et al., 1994; Burghardt et al., 1995; Carruba et al., 2002; Braet et al., 2003).

2.2.2. Data analysis

As described in chapter II, intercellular coupling in the sensory epithelium of the basilar papilla and utricular macula explants was examined by FRAP. Briefly, the fluorochrome calcein was introduced into live hair and supporting cells as its membrane-permeant acteoxymethyl (AM) ester form. An area of approximately 48 $\mu$m$^2$ within the sensory epithelium was bleached with maximum laser power and the recovery of fluorescence within the bleached region was subsequently monitored for 5 min with a greatly attenuated laser beam. The fluorescence intensity of calcein before and after photobleaching in selected regions of interest (ROIs) within hair and supporting cells was expressed in 8-bit grey-level units (from 0 to 255) as a function of time using LCS lite 2.0 (Leica). ROIs in the unbleached area served as a control for an overall decrease in fluorescence due to successive scanning and/or leakage of calcein to the medium (Fig. III.12C). The relative fluorescence recovery at time t was displayed as the percentage of prebleach levels

$$\text{PR}(t) = \left( \frac{I_r - I_0}{I_{pre}} \right) \times 100,$$

where $I_{pre}$, $I_0$, and $I_r$ were the measured fluorescence intensities of the ROI before (prebleach), at the first postbleach scan, and at the time t following the first scan, respectively (Stein et al., 1992; Stein et al., 1993).

The mean percentage recovery of 5 ROIs within supporting cells within a bleached area was sampled from different specimens to calculate the average fluorescence recovery for the apical and the basal regions of the basilar papilla, and the extrastriolar utricular macula. These fluorescence recovery curves were best fitted with either a biexponential function (Eq. 2) or a monoexponential function (Eq. 3).

$$\text{PR}(t) = I_{max 1} (1 - e^{-k_1 t}) + I_{max 2} (1 - e^{-k_2 t}),$$

$$\text{PR}(t) = I_{max} (1 - e^{-kt}),$$

where $I_{max}$ is the fluorescence intensity at its estimated diffusion equilibrium, expressed as a percentage of the prebleach level and k is the rate constant of the fluorescence recovery. As gap junction permeation is the rate-limiting step in the intercellular diffusion of the dye (Wade et al., 1986), the change of the dye concentration in the
bleached area with time \( \frac{dC_2}{dt} \) is proportional to the concentration gradient \( (C_1 - C_2) \) between the bleached and unbleached cells. This can be expressed by a modification of Fick's first law of diffusion as \( \frac{dC_2}{dt} = -P A \frac{(C_1 - C_2)}{V} \), where \( P \) is the permeability coefficient, \( A \) the gap junction area and \( V \) the cell volume. The rate constant \( k \) is related to the permeability coefficient by \( k = P A / V \) (Deleze et al., 2001).

Directionality of the dye spread was quantified by calculating the first derivative of the raw individual recovery curves for each ROI plot using the Savitzky-Golay smoothing method (5 points; Microcal Origin 5.0). A grid of ROIs was laid over the bleached area, with each ROI containing ~7,600 pixels (Fig. III.12D). The first derivatives were sampled according to their location within the basilar papilla, i.e. closer to the abneural/neural edge or proximal/distal end. In the utricular macula the striola and anterior/posterior part of the epithelium were used as reference points. The recovery rates at \( t_0 \) were expressed for each ROI as mean ± standard deviation (SD). Differences between the ROIs within a group were tested for statistical significance using a paired Student’s t-test.

### 2.2.3. Intercellular coupling in the sensory epithelia of the inner ear

Differences in the uptake, in the hydrolysis of calcein AM and/or in the cytoplasmic \( Ca^{2+} \) concentrations between hair and supporting cells led to a distinct pattern of brightly fluorescent hair cells surrounded by weaker fluorescent supporting cells. The bleaching at maximum power with the 488 nm laser line of an Argon-laser for 31 sec resulted in a decrease of fluorescence by \( 88 \pm 5\% \) (n=15) in the supporting cells of the basilar papilla and \( 92 \pm 3\% \) (n=7) in the utricular macula. The absence of propidium iodide within the bleached area indicated that bleaching had no obvious cytotoxic effects on the cells (Fig. III.12B). As shown by control regions (n=7) within the unbleached area, the decrease in overall fluorescence intensity owing to successive bleaching during the image acquisition and dye leakage was negligible (Fig. III.12C).

Five minutes after the photobleaching, the fluorescence had recovered only in supporting cells but not in hair cells (Fig III.13). To test whether calcein returned via gap junctions, FRAP experiments were conducted in the presence of the gap junction blocker carbenoxolone (CBX). No recovery of fluorescence within supporting cells was observed when the explants were incubated in 100 \( \mu M \) CBX for 30 min (n=5) (Fig III.14). By contrast, the inactive analogue GZA had no effect on the dye transfer.
Figure III.12: Methodology of FRAP. (A) Z-projection of the bleached area in a CBX-treated utricular macula. (B) Projection view of a utricular macula 30 min after bleaching. No significant increase of propidium iodide-labelled cells (red) was observed in the bleached area. (C) Quantification of fluorescence recovery in supporting cells. Changes in fluorescence intensity were measured in supporting cells (ROI 1,3,5) and hair cells (ROI 2,4,6) within the bleached area. ROI 7 in the unbleached area served as a control for overall bleaching. Fluorescence emission is plotted against time (major scale unit: 100 sec). (D) Quantification of spatial differences in the fluorescence recovery within the bleached area.
Figure III.13. FRAP in the auditory and vestibular sensory epithelium. Sequence of optical sections in (A) the basilar papilla and (B) the utricular macula taken before (prebleach) and immediately after bleaching (t = 0), and when the diffusion equilibrium of calcein is approached (t = 5 min). Fluorescence recovers only in supporting cells but not in hair cells (*). Bar = 20 μm.
Figure III.14. Blocking of gap junctional communication by CBX. FRAP in utricular macula explants incubated for 24 hours in 100 μM GZA (A) or CBX (B). Quantification of fluorescence recovery in supporting cells (ROI 1,2) and hair cells (ROI 3,4) within the bleached area revealed that no dye returned into supporting cells of CBX-treated supporting cells, in the absence of overall bleaching (ROI 5,6). Fluorescence emission is plotted against time (major scale unit: 50 sec).
Figure III.15. Contrasting of fluorescence recovery and Cx43/cCx31 expression in the supporting cells of the basilar papilla. (A) Perpendicular reconstruction of a Z-series through the basilar papilla taken 5 min after bleaching. The high fluorescence intensity in the lumenal region of supporting cells indicates that calcein returned predominantly through lumenal gap junctions. Bar = 20 μm. (A') Cx43 and cCx31-like immunostaining in a transverse section of the basilar papilla. Bar = 5 μm. Surface views of the (B) distal and (C) proximal regions of the basilar papilla taken 5 mins after bleaching and the corresponding projection views of Cx43 and cCx31-like gap junction plaques (B', C').
Quantitative analysis of gap-junction permeability

The permeability of gap junctions for calcein was quantified by estimating the rate constant $k$ and the percentage of maximal fluorescence recovery in the supporting cells of the basilar papilla and the utricular macula using Eq.2 and 3, respectively. The best fit for the mean recovery curves was monoexponential for the utricular macula, but biexponential for the basilar papilla (Fig. III.16).

The fluorescence intensity within supporting cells at its estimated equilibrium stayed well below prebleach levels, with 26% in the proximal region of the basilar papilla, 31% in the distal region of the basilar papilla, and 29% in the utricular macula. In the utricular macula, the estimated rate constant for calcein diffusion was $0.008 \text{ sec}^{-1}$, which resulted in a half time of 90 sec. In the basilar papilla, two components underlie the diffusion of calcein - a slow one similar to that found in the utricular macula and an initial fast one. The slow rate constants in the proximal and distal areas of the basilar papilla, by which 81-87% of the fluorescence recovered, were 0.008 and 0.007 sec$^{-1}$. But during the first 20 sec, diffusion followed a fast rate constant, which was 10 times faster than the slow component and by which 11% of the total fluorescence in the proximal and 16% in the distal end recovered.

Figure III.16. Fluorescence recovery curves of supporting cells.
The mean percentage of the recovery of the fluorescence intensity compared to prebleach levels within bleached supporting cells in the distal (□, $n=8$) and proximal (○, $n=8$) ends of the basilar papilla and the extrastriolar utricular macula (△, $n=5$) is plotted as a function of time. The solid line represents the fit to equation (2), the dashed line to equation (3).
### Table III.2 Transfer constants (k), equilibrium intensity (Ieq) and half times of fluorescence recovery for calcine in the distal and proximal basilar papilla and the extrastriolar utricular macula. The parameters are the averages (± SD) of the values obtained from the sampling of the mean percentage recovery curves of 5 separate regions within the supporting cells of a bleached area from n different specimens. The time by which half the fluorescence recovered (t1/2) was calculated as t1/2 = ln2/k. The goodness-of-fit is described as R².

<table>
<thead>
<tr>
<th></th>
<th>Basilar papilla (distal; n=8)</th>
<th>Basilar papilla (proximal; n=8)</th>
<th>Utricular macula (extrastriolar; n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>k₁ [sec⁻¹]</td>
<td>0.0066 ±0.0001</td>
<td>0.0075 ±0.0001</td>
<td>0.0078 ±0.0001</td>
</tr>
<tr>
<td>Ieq₁ [%]</td>
<td>25.68 ±0.12</td>
<td>23.05 ±0.07</td>
<td>28.64 ±0.12</td>
</tr>
<tr>
<td>t₁/2 [sec]</td>
<td>105.7</td>
<td>92.9</td>
<td>89.2</td>
</tr>
<tr>
<td>k₂ [sec⁻¹]</td>
<td>0.0609±0.0048</td>
<td>0.0842 ±0.0064</td>
<td></td>
</tr>
<tr>
<td>Ieq₂ [%]</td>
<td>4.78 ±0.21</td>
<td>2.88 ±012</td>
<td></td>
</tr>
<tr>
<td>t₁/2 [sec]</td>
<td>10.2</td>
<td>8.2</td>
<td></td>
</tr>
<tr>
<td>R²</td>
<td>0.999</td>
<td>0.999</td>
<td>0.997</td>
</tr>
</tbody>
</table>

**Asymmetric dye transfer in the basilar papilla**

In the utricular macula, calcine returned uniformly from all sides to the bleached area, but in the basilar papilla fluorescence recovered predominately from the abneural side of the square to the neural side (for the movie see CD in the back cover). This asymmetric dye transfer was not caused by scanning artefacts, as the rotation of the specimen by 90° altered the point of entry of the dye by the same degree (n=3).

To validate the asymmetric dye transfer, the change of fluorescence intensity immediately after photobleaching (dl/dto) was calculated for six ROIs. Random diffusion would result in similar recovery rates at the borders of the bleached area, slowing down towards the centre. Directional diffusion, however, would produce recovery rates that decrease across/along the bleached area. As shown in Fig. III.17, there were significant differences in the recovery rates between the abneural and neural edges of the bleached square in the distal (p<0.001) and proximal (p<0.05) basilar papilla, although not between its proximal and distal sides. However, no significant differences in the recovery rates were measured in either the striolar/extrastriolar or the anterior/posterior direction within the utricular macula.

Differences in recovery were not only observed within an optical section but also within the depth of the sensory epithelium (Fig. III.15A). Preliminary data for the distal basilar papilla indicated that fluorescence recovered faster in ROIs near the lumenal section that the ROIs closer to the basilar membrane (Fig. III.17D).
Figure III.17. Quantification of the spatial patterns of fluorescence recovery.

The mean derivatives (± standard deviation) of the fluorescence recovery within an optical section are plotted for 6 ROIs covering the bleached area in (A) the distal basilar papilla (n=11), (B) the proximal basilar papilla (n=11), and (C) the extrastriolar utricular macula (n=9). (D) The mean derivatives (n=3) of the fluorescence recovery within the depth of the distal basilar papilla. The schematic illustration indicates the location of the bleached areas (black squares) within the sensory epithelia. The grey strip in the utricular macula marks the striola. (*) indicates significant differences in the fluorescence recovery between the neural and abneural regions within the basilar papilla (paired Student’s t-test; p<0.05).

3. Discussion: Methodology

3.1. Immunohistochemistry

Despite the lack of specific antibodies to cCx31, this avian connexin isoform was detected using antibodies to mammalian Cx26 and Cx30. Several lines of evidence strongly point to the cross-reactivity of Gap 28H and 33-2500 with cCx31: 1) the sequences of immunogenic peptides of both antibodies share a high homology with the correlating cCx31 sequence; 2) Gap 28H and 33-2500 detect gap-junction plaques...
between neighbouring HeLa cells transiently transfected with cCx31; and 3) both antibodies recognise the same protein band at 31 kDa in Western blots. The lack of protein bands with a migration profile between 20 and 30 kDa further suggests that no other orthologues to mammalian Cx26 and Cx30 are expressed in the inner ear of the chick. However, due to the close proximity of the molecular weights of mCx30 and cCx31, it cannot be completely ruled out that the two protein bands were not resolved in Western blots and that an orthologue to mCx30 may be present in the avian inner ear.

The distribution pattern of immunolabelled gap junctions was consistent with previous freeze-fracture studies of gap junctions in the avian inner ear (Forge et al., 2003a) in that the plaques were smaller at the luminal region of supporting cells compared with their basal surfaces. The size of the basal junctions measured in freeze-fracture replica was similar to that found by immunohistochemistry, with junction sizes up to 8 \( \mu m^2 \) in the basilar papilla, and up to 4 \( \mu m^2 \) in the utricular macula. However, immunofluorescence showed in general fewer, but larger (~2 \( \mu m^2 \)), luminal plaques than freeze fracture (~0.17 \( \mu m^2 \)). Although valid for comparative studies, the number and average area of the immunolabelled gap junctions can be regarded only as crude estimates, as the limited resolution of the confocal microscope and the software used for the quantification do not allow for accurate measurement of closely packed particles. This inaccuracy, which causes the dense packing of gap junctions to appear as fewer but larger plaques, is thus likely to be the cause for the differences between freeze fracture and immunofluorescence studies.

The gap-junction pattern observed in chick hatchlings is likely to resemble the connexin expression in the adult animals. The basilar papilla of these precocious animals is thought to mirror closely adult morphology and physiology at the time of hatching. Although the length of the basilar papilla subsequently increases (Ryals et al., 1984), chicks hatch with a complete number of fully developed hair cells (Tilney et al., 1986), whose tonotopic organisation and tuning properties change only insignificantly as the animal matures (Manley et al., 1987; Manley et al., 1991). The potassium concentration of the endolymph and the endochlochlear potential are mature at the time of hatching (Runhaar et al., 1991), despite the continuing differentiation of the tegmentum vasculosum and dark cells for another seven days (Cotanche et al., 1987). In contrast to the basilar papilla, the number of hair cells increased in the utricular macula during the first 16 weeks after hatching as new cells continue to be produced by asymmetric cell division (Goodyear et al., 1999). However, the overall hair cell density
and hair cell/supporting cell ratios at post hatched day 2 are comparable to the mature utricular macula due to a non-linear increase in the surface area of the epithelium.

3.2. Fluorescence recovery after photobleaching

Fluorescence recovery after photobleaching is a relatively simple and non-invasive way of examining dye spread in the intact epithelia of the avian inner ear. Confocal microscopy permits the examination of dye spread in a three dimensional tissue not only within an optical section but also within its depth. The extension of the bleached area from one cell to an array of cells enabled the examination of differences in the spatial and temporal diffusion patterns within the supporting cell syncytium.

The quantification of gap junction permeability based on fluorescence recovery has to be regarded as a simplistic and crude estimate of the real permeability, as diffusion in a tissue is a complex process dependent on a multitude of parameters. Quantitative analysis of recovery rates usually assumes that all cells have the same volume and are equally coupled. Even in such idealistic circumstances, diffusion is dependent on the general mobility of the dye within the cytoplasm, the junctional permeability of the dye, and the leakage of the dye across the plasma membrane. Dye transfer in a three dimensional epithelium is influenced not only by intrinsic properties of the fluorescent tracer but variations between the coupled cells, such as differences in volume, metabolism and health of the cells. The heterogeneity of the distribution and area of gap junctions, as well as differences in the number of gap junction channels and their connexin composition, strongly affect the rate constant.

The recovery of calcein, reflected by the fluorescence intensity, was 26-31% of prebleach levels. As calcein is a relative large and polyanionic dye (623 Da, -4), its mobility through the cytoplasm and permeability through the densely packed gap junction channels is likely to be hindered and may account for the low dye return. In addition, the prebleach intensity levels in supporting cells may have been inflated owing to the possible incorporation of pixels from saturated hair cells into the ROIs along the hair cell/supporting cell borders.

As expected for diffusion along a concentration gradient, the fluorescence recovery curves followed an exponential time course. However, the best fits for the recovery curves differed between basilar papilla and utricular macula and, as will be described in chapter IV, even between the normal and gentamicin-treated basilar papilla. In the utricular macula (and the basilar papilla following hair cell loss) the recovery curve was monoexponential, whereas it was biexponential in the basilar papilla. There are several
reasons that might cause the biexponential recovery curve in the normal basilar papilla. First, the hydrolysis of calcein AM might produce a fluorescent by-product with a faster rate constant than calcein. However, this is unlikely as such a compound would have been exclusively produced in the (undamaged) basilar papilla. Secondly, the fast constant rate may have been caused by recovery of fluorescence from intracellular calcein reservoirs owing to the insufficient bleaching in the basilar papilla. This scenario is unlikely as the fast constant rate is absent in gentamicin-treated basilar papillae. Thirdly, the biexponential recovery may be caused by the presence of connexin-isoforms with different permeabilities for calcein. Considering that the major difference between the basilar papilla and the utricular macula (and gentamicin-treated basilar papilla) is the expression of Cx43 in the sensory epithelium, it is tempting to speculate that Cx43 has a 10-fold higher permeability for calcein than cCx31 and so underlies the fast diffusion constant.

Gap junctional communication in the basilar papilla and the utricular macula differed not only in its temporal kinetics but also in its spatial kinetics. While calcein spread uniformly in the utricular macula, it recovered predominantly from the abneural/distal direction in the basilar papilla. As stated earlier, the asymmetric dye transfer was not caused by scanning artefacts, as the rotation of the specimen by 90° altered the point of entry of the dye by the same degree. The first derivatives of the recovery curve within a grid of ROIs were calculated, as it enabled a standardised way of analysing the spatial component of dye return. The inclusion of hair cells into the ROI, however, is not ideal, as the absence of fluorescence recovery in the sensory cells decreases the overall rate of recovery in a given ROI. For a quantitative comparison of the first derivatives, the different percentages of hair cell on the bleached area have to be taken into account.
IV. GAP JUNCTIONS DURING HAIR CELL REGENERATION

1. Introduction: Hair Cell Regeneration in the Avian Inner Ear

Hair cell regeneration in the inner ear was first identified in the mid 1980s in the basilar papilla of chickens. Studies aimed at examining hair cell damage following acoustic trauma (Cotanche, 1987) and aminoglycoside antibiotics (Cruz et al., 1987) reported that new hair cells were formed in areas of hair cell lesions. Subsequent studies revealed that lost hair cells were mainly replaced by cell division within the damaged basilar papilla as both hair and supporting cells were labelled with markers for DNA synthesis (Corwin and Cotanche, 1988; Ryals and Rubel, 1988). This discovery was unexpected since it was assumed that the postembryonic production of hair cells in vertebrates was restricted to anamniotes, such as fish and amphibians (Corwin, 1981; Corwin, 1985). It is now well established that, in response to hair cell loss, supporting cells in the basilar papilla enter the cell cycle, proliferate and subsequently differentiate into new, functional hair cells (reviewed by Cotanche et al., 1994; Corwin and Oberholtzer, 1997; Cotanche, 1997; Cotanche, 1999; Smolders, 1999; Stone and Rubel, 2000; Bermingham-McDonogh and Rubel, 2003). In contrast to the basilar papilla, the postembryonic vestibular epithelia produce hair cells continuously (Jorgensen and Mathiesen, 1988), which is thought to occur as a result of the short life span of vestibular hair cells (Kil et al., 1997; Goodyear et al., 1999). Experimentally induced hair cell loss provokes the increase of the proliferation rate of vestibular supporting cells (Weisleder and Rubel, 1992; Carey et al., 1996; Matsui et al., 2000).

Two principal methods have been used to induce hair cell loss in birds - exposure to intense sound and ototoxic antibiotics (reviewed by Cotanche et al., 1994; Cotanche, 1999). Pure tones, third octave noise or octave band noise (sound level between 100-140 dB) cause hair cell loss along the basilar papilla and the uncoupling of the tectorial membrane from surviving hair cells. The location and extent of the lesion, which is usually confined to a crescent-shaped area of abneural short hair cells, is determined by the frequency and intensity of the stimulus (Ryals and Rubel, 1982; Cotanche et al., 1987). Aminoglycosides (e.g. gentamicin, kanamycin, and streptomycin), administered by injection or local application, gradually destroy both types of hair cells, beginning in the proximal region of the basilar papilla and the striola in the utricular macula. Aminoglycoside-trauma is confined to hair cells and does not cause direct damage to accessory structures, such as the tectorial membrane.
Structural damage and excitotoxicity (Pujol and Puel, 1999) are regarded as the predominant causes of cell death following acoustic trauma. The ototoxicity of aminoglycoside may be caused by oxidative stress associated with the formation of free radicals (Priuska and Schacht, 1995) and excitotoxicity (Basile et al., 1996). Morphological evidence and the detection of caspase pathways identified apoptosis (programmed cell death) as the main process of hair cell death (Li et al., 1995). The majority of dead hair cells are extruded from the sensory epithelium, while some injured hair cells may be phagocytosed by macrophages (Warchol, 1997). Parallel to the death and extrusion of hair cells, the apical surfaces of supporting cells expand (Cotanche and Dopyera, 1990) and close the lesion in order to preserve the integrity of the ionic compartments of scala media and scala tympani without disrupting the reticular lamina.

Hair cell death triggers an as yet unidentified mitogenic event, which causes supporting cells to enter the cell cycle. Virtually all supporting cells leave growth arrest (G0) and enter G1, however only supporting cells in the proximity of hair cell damage reach the DNA synthesis phase (S) (Bhave et al., 1995). The first entry of supporting cells into the S phase of the cell cycle is detected 12-24 hours after the onset of the damaging stimulus (Stone and Cotanche, 1994; Warchol and Corwin, 1996) and the level of supporting cell proliferation reaches its maximum within 2-3 days before returning to pre-damaged levels after 5-7 days (Hashino and Salvi, 1993; Bhave et al., 1995; Stone et al., 1999; Matsui et al., 2000).

The transition from G0 to S phase is generally initiated by extracellular factors, such as soluble mitogens, cytomechanical changes and interaction with the extracellular matrix (reviewed by Pardee, 1989; Huang and Ingber, 1999). A variety of studies suggest that hair cell death triggers the release of a diffusible mitogen from within the sensory epithelium (Warchol and Corwin, 1993; Tsue et al., 1994; Warchol and Corwin, 1996). Among the candidates for the putative trigger are fibroblast growth factors (FGF) (Zheng et al., 1997) and insulin-like growth factors (IGF) (Oesterle et al., 1997). These mitogens bind to tyrosine receptor kinases on target cells, thereby initiating an intercellular signalling cascade that culminates in DNA synthesis and cell division. Possible cell signalling pathways include the PI3-K/TOR and MAPK pathways which have been shown to increase proliferation in the utricular macula in chickens (Witte et al., 2001) and mice (Montcouquiol and Corwin, 2001). Furthermore, the activation of cAMP signalling increased the proliferation of supporting cells in the damaged and undamaged basilar papilla (Navaratnam et al., 1996) and mammalian utricular macula (Montcouquiol and Corwin, 2001). In addition, growth factors and cytokines, such as
interleukin-1β and tumour necrosis factor α (Warchol, 1999), may be secreted locally by macrophages, which have been reported to migrate to sites of hair cell lesions in the avian basilar papilla (Warchol, 1997) and utricle (Bhave et al., 1998). Given that hair cell regeneration requires the loss of hair cells, it is likely that antimitotic stimuli, in addition to stimulating factors, regulate supporting cell proliferation. The fibroblast growth receptor 3 (FGFR-3) (Bermingham-McDonogh et al., 2001), the basic fibroblast growth factor (FGF-2) (Oesterle et al., 2000), and retinoic acid (Warchol, 2002) may be involved in the inhibition of cell proliferation in the avian inner ear, although an conclusive signalling pathway has yet to be identified.

In addition to mitogens, an interaction of cell density, contact mediated signals and the composition of the extracellular matrix may play a further part in the regulation of supporting cell proliferation. The extrusion of hair cells temporarily disrupts tight and adherens junctions between hair and supporting cells, and this might activate putative signalling molecules located at those junctions. N-cadherin, a calcium-dependent adhesion molecule, which is linked to the cytoskeleton via β-catenin, has been detected in the avian auditory and vestibular epithelium (Warchol, 2002). It has been proposed that free β-catenin, a regulatory molecule that is involved in the Wnt signalling pathway, stimulates entry into the cell cycle by interacting with the T cell factor (TCF) transcription factors. In addition, the expansion of supporting cell surfaces is likely to change the arrangement of the cytoskeleton, which has been shown to influence the proliferation of many types of cells (Huang and Ingber, 1999).

Once division of supporting cells has created a pool of undifferentiated cells, the progenitor cells have to differentiate into hair and supporting cells. As early as three days after injury, immature hair cells begin to appear in the lesion sites. The pattern of the hair cell-supporting cell mosaic suggests that hair cell differentiation occurs via lateral inhibition, whereby a cell adopting a particular fate inhibits its immediate neighbours from doing likewise (Goodyear and Richardson, 1997; Pickles and van Heumen, 2000). There is initial evidence that Delta-Notch signalling, which mediates lateral inhibition, may be involved in the patterning of the regenerated sensory epithelium (Stone and Rubel, 1999).

In addition to stimulation of proliferation, there is emerging evidence that supporting cells convert directly into hair cells. This could account for apparently non-mitotic productions of hair cells. Direct transdifferentiation was first identified in the bullfrog (Baird et al., 1993) but has since been reported in the avian basilar papilla (Adler and Raphael, 1996; Adler et al., 1997) and the mammalian vestibular epithelium,
where it appears to be a major pathway for the production of new hair cells following aminoglycoside damage (Rubel et al., 1995; Li and Forge, 1997; Forge et al., 1998). Some studies have also suggested that sublethally damaged hair cells may be capable of self-repairing (Sobkowicz et al., 1996; Sobkowicz et al., 1997; Gale et al., 2002).

Behavioural and physiological studies confirmed that regeneration of hair cells restores near normal hearing and balance (reviewed by Smolders, 1999; Bermingham-McDonogh and Rubel, 2003). The auditory and vestibular nerve fibres, which are left intact after both types of traumata (Duckert and Rubel, 1990; Ofsie and Cotanche, 1996; Hennig and Cotanche, 1998), grow back and form synapses with the new hair cell, and functional transduction mechanisms and gradients of ion channels responsible for electrical tuning are re-established. The functional recovery has been mainly studied in the auditory system, in which recovery after acoustic trauma is rapid and nearly complete. The rapid recovery appears to be associated with the repair of surviving hair cells and accessory structures rather than the regeneration of hair cells. The recovery after aminoglycoside damage is slower and mirrors the structural regeneration of hair cells more closely. Permanent functional deficits remain in the regenerated areas, which are most likely associated with malfunction of electrical tuning of hair cells and/or synaptic connections.

2. Results: Gap Junctions during Hair Cell Regeneration

As stated earlier, gap junctions have been implicated in the coordination of cell proliferation and differentiation. It is therefore possible that gap junctions may be involved in hair cell regeneration by conveying putative mitotic and/or inhibitory signals, which are known to act over a distance (Warchol and Corwin, 1996). To explore the role of gap junctions during hair cell regeneration, changes in the connexin expression were assessed in organotypic cultures of basilar papilla and utricular macula and the effects of the pharmacological blocking of gap junctions on the proliferation of supporting cells were examined.

2.1. Hair Cell Loss and Regeneration in Organotypic Cultures

Hair cell loss and regeneration in organotypic cultures of the basilar papilla and the utricular macula was qualitatively assessed using scanning electron microscopy (SEM) and immunohistochemistry. The progression of hair cell loss was examined in cryosections of basilar papilla explants, which were incubated for 6, 12 and 24 hours in
1 mM gentamicin. Hair cell death was monitored by labelling the cytoplasms of hair cells with the specific hair cell-antibody HCS-1 (Finley et al. 1997) and the nuclei with DAPI (Fig. IV.1). In control tissue, the somata and nuclei of the hair cells were aligned at the lumenal surface of the epithelium, whereas the supporting cell nuclei were located near the basilar membrane (Fig. IV.1A). Six hours after the addition of gentamicin to the medium, extensive damage to the hair cells was visible, extending from the proximal to the distal tips of the basilar papilla. Apoptotic hair cells, characterised by their shrunken, rounded cell bodies and their condensed (pyknotic) nuclei, were detected within the epithelium (Fig. IV.1B). Twelve hours after the exposure to gentamicin, almost all hair cells were extruded from the epithelium along the whole length of the basilar papilla (Fig. IV.1C).

The extent of hair cell death was confirmed by SEM, as virtually no hair bundles were observed in the basilar papilla (Fig. IV.2B) and in the utricular macula (Fig. IV.3B) one day after the gentamicin-treatment (D1). Regenerated hair cells, identified by their immature hair bundles, were visible in both epithelia from D3 onwards. At D5, immature hair bundles had a tuft-like appearance, due to the similar length of the stereocilia that covered the hair cell surface. In addition, a kinocilium was present behind the prospective tallest row (Fig. IV.2&3C). The maturation of the regenerated hair cells was followed by double labelling wholemount preparations with two hair cell-specific antigens, HCS and HCA, the latter being associated with the surfaces of stereocilia (Goodyear and Richardson 1992). HCA/HCS-positive cells were detected across the width of the distal tip at D3 (Fig. IV.4B). At this stage, the hair cells had elongated somata, making contact with both the lumenal surface and basilar membrane. By D7, the majority of hair cells had lost this supporting cell characteristic and were confined to the lumenal surface of the epithelium (Fig. IV.4C).

Figure IV.2. & 3. Hair cell loss and regeneration in basilar papilla and utricular macula explants. SEM micrographs of the distal basilar papilla and the extrastriolar region of the utricular macula. (A) In control explants, the hair cells (HC) are arranged in a hexagonal pattern and surrounded by supporting cells (SC). (B) 1 day after the 24 hour incubation in 1 mM gentamicin virtually all hair cells are extruded (arrowhead), apart from few immature hair cells (arrows) in the utricular macula, which were already present at the time of gentamicin-application and are unaffected by its ototoxicity. The lesions are closed by the expansion of supporting cells, whose surfaces are covered with microvilli and a central kinocilium is visible. (C) 5 days after gentamicin treatment immature hair bundles (arrows) at different stages of hair bundle maturation are visible. Bar = 5 μm.
Figure IV.1. Progression of gentamicin-induced hair cell death in basilar papilla explants. Labelling of hair cells (HCS; green), nuclei (DAPI; blue) and F-actin (phalloidin; red) in cryosections of the distal basilar papilla. (A) Explant cultured for 24 hours in control medium. THC, tall hair cell; SHC, short hair cell; SC, supporting cell; TM, tectorial membrane. (B) 6 hours and (C) 24 hours after the addition of 1 mM gentamicin to the medium. Arrows indicate shrunken hair cell bodies with pyknotic nuclei within the epithelium; arrowheads mark extruded hair cells, trapped between the tectorial membrane and the sensory epithelium or embedded in the newly excreted tectorial membrane. Bar = 20 μm.
Figure IV.2. Hair cell loss and regeneration in basilar papilla explants.
Figure IV.3. Hair cell loss and regeneration in utricular macula explants.
Figure IV.4. Maturation of regenerated hair cells in basilar papilla explants. Z-projections of HCS/HCA-labelled hair cells in the distal region of the basilar papilla. (A) In the control explant cultured for 5 days, hair cells are tightly packed and confined to the lumenal surface of the epithelium. (B) 3 days post-gentamicin, HCS/HCA-positive cells are visible across the epithelium and many cells have descending processes (arrows). (C) 7 days post-gentamicin, the majority of the labelled hair cells have rounded cell bodies and are confined to the lumenal region of the epithelium. Bar = 20 μm.
2.2. Connexin Expression following Hair Cell Loss

The expression of Cx43 and cCx31 following aminoglycoside-induced hair cell loss was qualitatively examined by immunohistochemistry in cryosections of organotypic cultures of the basilar papilla. The expression pattern of Cx43 and cCx31 in undamaged control cultures closely resembled that of the *in vivo* basilar papilla (Fig. IV.5). cCx31-like immunostaining was detected in the supporting cells, in the tegmentum vasculosum, in homogene cells, clear cells, hyaline cells, and cuboidal cells. Cx43-immunoreactivity was confined to the supporting cells of the basilar papilla, where it extensively overlapped with cCx31-like labelling. To follow the expression patterns of Cx43 and cCx31 during hair cell loss, organotypic cultures were fixed 6, 12 and 24 hours after gentamicin was added to the medium (Fig. IV.5). Six hours after the addition of gentamicin, Cx43 labelling was concentrated at the apical surface of supporting cells. Below the apical surface, immunoreactivity for Cx43 decreased, paralleling a decline of the co-localisation of Cx43 and cCx31. After 12-24 hours virtually no immunoreactivity for Cx43 could be detected in the sensory epithelium. One day post gentamicin, Cx43-staining re-appeared at the apical surface of the supporting cells. In contrast to the large sheet-like plaques in the control tissue, the re-emerging Cx43 plaques were small and dot-like (Fig. IV.6). By D3, the size of the Cx43-positive plaques slightly increased and plaques became visible in the depth of the tissue. Furthermore, some co-localisation with cCx31-like plaques was detected at that time point. The pre-damaged expression pattern was not recovered by D7, by which time Cx43 expression is likely to have been influenced by the general deterioration of the tissue.

The changes in the expression pattern of cCx31 were less dramatic and were likely to have been caused by normal variation within the cultured tissues. However, there appeared to be a tendency for fewer, though larger, cCx31-like gap junction plaques immediately following the 24 hours of gentamicin exposure (Fig. IV.5). From D1 onwards, a decrease in the density of cCx31-like plaques was apparent along the neural edge of the basilar papilla, while the abneural half of the epithelium remained extensively stained (Fig. IV.6).

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Figure IV.5 & IV.6. Connexin expression after hair cell loss and during hair cell regeneration. Double labelling of cryosections of distal basilar papilla explants with a monoclonal Cx30 antibody (33-2500; FITC) and a polyclonal Cx43 antibody (C6129; Alexa Fluor 633). The F-actin rich hair bundles are labelled with TRITC-conjugated phalloidin. (A) Explant cultured for 5 days in control medium. (B) 6 hours after gentamicin addition to the medium. (C) Explant cultured for 5 days in control medium. (D) 24 hours after gentamicin addition. (D) 1 day after the 24 hour gentamicin treatment. (E) 3 days after gentamicin treatment. (F) 7 days after gentamicin treatment. Bar = 20 μm.
Figure IV.5. Connexin expression after hair cell loss.
Figure IV.6. Connexin expression during hair cell regeneration.
Figure IV.7. FRAP following hair cell loss. Optical sections of (A) an undamaged basilar papilla cultured for 2 days and (B) a basilar papilla incubated in gentamicin for 24 hours, which caused complete hair cell loss and the expansion of supporting cells. The images were taken immediately before and 5 mins after the bleaching of calcine in a defined region of the epithelium (dashed square). Bar = 20 μm.

Figure IV.8. Comparison of the instantaneous fluorescence recovery rates between control and gentamicin-treated basilar papilla explants. The mean recovery rates (± SD) are calculated for 6 different regions of interest within the bleached area, which describe the abneural-neural and distal-proximal axes of the basilar papilla. As hair cells do not contribute to fluorescence recovery, the rates in the drug-damaged epithelia were reduced by 65%, which corresponds to the hair cell area within a ROI located at the distal end of the basilar papilla.
2.3. **Intercellular Coupling following Hair Cell Loss**

To examine whether the downregulation of Cx43 affected the intercellular coupling of supporting cells, FRAP experiments were performed on basilar papilla explants following a 24 hour exposure to gentamicin (Fig. IV.7). Fluorescence recovered in both control and gentamicin-treated basilar papillae, but with different spatial and temporal patterns. In the control epithelia (n=4), cultured for 2 days, fluorescence recovered in a biexponential manner, similar to the 1-day cultures described in chapter III. However the amount of dye transfer and the fast rate constant were markedly decreased in comparison with 1-day cultures. The intensity at its diffusion equilibrium was estimated to be 20.79 ±4.11% of the prebleach level, of which 17.59 ±1.56% recovered through the slow rate constant at 0.0067 ±0.0016 sec⁻¹. While 3.20 ±2.55% recovered through the fast rate constant at 0.0326 ±0.0161 sec⁻¹. By contrast, in the drug-damaged epithelia (n=8) fluorescence recovered in a monoexponential time course; 50.16% ±2.12% of prebleach levels recovered at a rate of 0.0034 ±2.12 sec⁻¹, which resulted in a half time of 203.9 sec.

No asymmetric dye transfer was observed in gentamicin-treated basilar papilla explants. This was confirmed by calculating the instantaneous recovery rates for 6 ROIs within the bleached area (Fig. IV.8). Whereas the control basilar papillae showed a decrease in the rate of fluorescence recovery from the abneural to the neural edge of the bleached square, no such decline was observed in the sensory epithelia following hair cell loss. No differences between the distal and proximal-facing sides of the bleached area were observed in both control and drug-damaged papillae.

2.4. **Pharmacological Blocking of Gap Junctions**

To examine the possible involvement of gap junctions in hair cell regeneration, the level of supporting cell proliferation following gentamicin-induced hair cell loss was assessed in the presence of the unspecific gap junction blocker, carbenoxolone (CBX), and its inactive analogue, glycyrrhizinic acid (GZA).

2.4.1. **Glycyrrhetinic acid and its derivatives**

A major impediment in the study of the functional role of gap junctions is the absence of specific blockers that completely close gap junction channels without further side effects. This has been partly overcome by generating gap junction-specific uncoupling agents in the form of connexin-specific antibodies or mimetic peptides.
Antibodies, raised against intra- and extracellular domains of connexins, bind directly to the exposed sequences and have been shown to block gap junctional conductance and dye transfer. Mimetic peptides simulate crucial extracellular loop sequences, thereby disrupting the docking of connexons and/or channel gating (reviewed by Evans and Boitano, 2001). In addition, antisense oligonucleotides have been used to transiently block the translation of specific connexin-isoforms by binding to its messenger RNA via Watson-Crick base pairing (Moore and Burt, 1994; Green et al., 2001).

The majority of functional studies of gap junctions, however, rely on relatively indiscriminate and often toxic uncoupling agents (reviewed by Rozental et al., 2001). Classical inhibitors of gap junctions, include octanol, heptanol and the anaesthetic halothane, all of which induce localised changes in membrane fluidity that lead to the unspecific modulation of gap junctions and other ion channels. An uncoupling agent, with a relatively high potency and only weak adverse effects on the viability of the cells, is glycyr rhetinic acid and its derivatives.

Glycyrrhetinic acid (GA) is an aglycone of glycyrrhizic acid, which is extracted from licorice (*Glycyrrhiza glabra*) roots. The α- and β-stereoisomers of 18-glycyrrhetinic acid and their synthetic analogue, carbenoxolone (CBX), have been successfully used to study the role of gap junctional communication, e.g. in neuronal differentiation (Bani-Yaghoub et al., 1999) and fibroblast growth control (Martin et al., 1991). Transfer of fluorescent dyes is almost completely and reversibly inhibited by GA derivatives. Electrical coupling is decreased in a dose-dependent manner about fourfold, but never completely inhibited. The precise mechanisms of the inhibition are not clear, but it has been proposed that GA derivatives obstruct intercellular coupling by binding directly to the connexons, inducing a conformational change that results in the closure of the channel (Davidson and Baumgarten, 1988). CBX-blocking is associated with the disruption of the connexon arrangement within gap junction plaques, causing an approximately 30% decrease in connexon density (Goldberg et al., 1996).

In addition to their actions on gap junctions, GA derivatives are known to interact with mineralcorticoid and glucocorticoid receptors and to inhibit the enzyme 11β-hydroxysteroid dehydrogenase, which converts cortisol into the inactive cortisone (Walker and Edwards, 1994). Furthermore, liquorice derivatives are widely used in the treatment of gastric ulcers (Langman, 1977) and have been shown to exhibit anti-inflammatory and even anti-viral activities (Pompei et al., 1979; Tochikura et al., 1989).
2.4.2. Data analysis

The quantification of BrdU-labelled cells was carried out on wholmount preparations of basilar papilla and utricular macula explants 1 and 3 days after gentamicin-treatment (D1, D3) and their undamaged controls (3d, 5d). Montages were assembled from z-axis projections of optical sections confined to the sensory epithelium using Photoshop 6.0 (Adobe). Montages containing only BrdU-labelled nuclei were imported as greyscale images into ImageJ 1.31 (NIH), where the pixel intensity thresholds were interactively set. Overlapping nuclei were automatically separated using the "watershed" feature of the software. To exclude small debris and non-incorporated BrdU from the quantification, only particles larger than 20 pixels were counted within the ROI. In the utricular macula, the ROI demarked the entire sensory epithelium. In the basilar papilla, the ROI was confined to the distal half of the sensory epithelium as any proliferation at the proximal end was likely to have been caused by surgical trauma. The automatic quantification was corrected by hand on printouts of the counted nuclei. To calculate the number of BrdU-labelled cells/mm², the area of the epithelia was measured in greyscale images of propidium iodide labelling. The data of 3d/D1 samples were compared using a one-way ANOVA with the Bonferroni post test (GraphPad Instat). No statistical analysis was performed for 5d/D3 specimens due to their small sample size.

2.4.3. Pharmacological blocking and cell viability

Incubation of basilar papilla and utricular macula explants in 100 μM CBX for a minimum of 30 min caused a decrease in gap junctional communication, which resulted in a 70-80% reduction of calcein transfer between supporting cells (Fig IV.9). The incubation in 100 μM CBX had no adverse effects on hair and supporting cells for at least two days. Both cell types metabolised calcein AM and their nuclei exhibited normal morphology (Fig. IV.10A). However, prolonged incubation in CBX (from 5 days onwards) increased hair cell death as indicated by the presence of deformed and pyknotic nuclei in the hair cell layer of the epithelium (Fig. IV.10B). No obvious adverse effects of CBX on supporting cells were observed, which showed not only a dense packing of their nuclei but also that they were still able to synthesize their DNA.

2.4.4. Supporting cell proliferation

The effects of the CBX-induced decrease in gap junctional communication on supporting cell proliferation were examined by BrdU-immunohistochemistry. The quantification of cells in their S-phase was carried out on organotypic cultures at D1 and D3, which were incubated in BrdU for 6 hours prior to fixation.
Figure IV.9. Comparison of the maximal fluorescence recovery between GZA and CBX-supplemented basilar papilla and utricular macula explants. Mean percentages of prebleach levels are plotted for GZA cultures of basilar papillae (n=5) and utricular maculae (n=3), and CBX-treated explants (n=3).

Figure IV.10. Cell viability in CBX-treated explants. Propidium iodide (red)- and BrdU (green)-labelling in the hair cell and supporting cell layer of utricular macula explants cultured for (A) 2 days and (B) 5 days in 100 μM CBX. Nuclei in the supporting cell layer are round and densely packed at both time points, while pyknotic hair cell nuclei (arrows) are visible at day 5, which may account for the increase in BrdU-labelled cells. Bar = 20 μm.
Figure IV.11. Proliferation pattern in GZA and CBX-supplemented explants of the utricular macular.
The 6 hour BrdU-pulse resulted in the incorporation of BrdU in a sufficient but countable number of nuclei, which resembled the progression and pattern of proliferation observed in explants that were continuously incubated in BrdU. In the basilar papilla, the proliferation of the supporting cells was confined to the neural edge of the epithelium and progressed over time from its distal to its proximal ends. In the utricular macula, proliferating cells were observed throughout the epithelium, but were concentrated in the striolar region (Fig. IV.11).

**Quantitative analysis of supporting cell proliferation**

**Utricular macula.** There was no significant difference in the number of BrdU-labelled cells observed in undamaged explants of 3-day-old (3d) GZA- and CBX-supplemented cultures (Fig IV.12A). However, there was a significant difference (p<0.01) in the number of BrdU-labelled cells after the cultures were treated with gentamicin. At 1 day post-gentamicin (D1), CBX-supplemented explants had only half the number of BrdU-labelled nuclei as did GZA cultures.

<table>
<thead>
<tr>
<th>Utricular macula</th>
<th>GZA</th>
<th>CBX</th>
</tr>
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<tbody>
<tr>
<td>3d control (D1)</td>
<td>703.5 ±158.8 (n=5)</td>
<td>598.7 ±148.7 (n=5)</td>
</tr>
<tr>
<td>1d post-gentamicin (D1)</td>
<td>1867.6 ±347.0 (n=4)</td>
<td>969.5 ±459.1 (n=5)</td>
</tr>
<tr>
<td>5d control (D3)</td>
<td>179.1 ±23.9 (n=3)</td>
<td>1229.8 ±190.1 (n=2)</td>
</tr>
<tr>
<td>3d post-gentamicin (D3)</td>
<td>835.5 ±50.2 (n=2)</td>
<td>396.8 ±26.2 (n=2)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Basilar papilla</th>
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<tbody>
<tr>
<td>3d control (D1)</td>
<td>4.67±3.05 (n=3)</td>
<td>5 ±3.6 (n=3)</td>
</tr>
<tr>
<td>2d post-gentamicin (D1)</td>
<td>367.6 ±124.9 (n=5)</td>
<td>169.3 ±158.8 (n=6)</td>
</tr>
<tr>
<td>3d post-gentamicin (D3)</td>
<td>136.0 ±97.6 (n=2)</td>
<td>39.7 ±7.2 (n=3)</td>
</tr>
</tbody>
</table>

Table IV.3. Comparison of the number of BrdU-labelled cells/mm² (±SD) in control and gentamicin-treated explants of utricular macula and basilar papilla that were cultured in GZA or CBX-supplemented medium.

By day 5 (5d), the numbers of DNA synthesising cells in GZA-supplemented control cultures markedly decreased, while in the CBX-supplemented controls the number of proliferating cells almost doubled. As noted earlier, this large increase in BrdU-labelled cells in the CBX control cultures at 5d is likely to be caused by the increased death of hair-cells rather than the blocking of gap junctions.

Figure IV.11. Proliferation pattern in GZA and CBX-supplemented explants of the utricular macula. Binary images of BrdU-labelling in the utricular macula at different time points during hair cell regeneration. Arrows indicate a strip within the striola, which lacks BrdU-positive cells. Bar = 250 mm.
As with D1, by 3 days post-gentamicin (D3) there was a pronounced difference in the number of BrdU-labelled cells between the two culture systems, with a similar ratio of proliferating cells being observed in CBX-supplemented explants compared with GZA cultures. Additionally, GZA-supplemented gentamicin-damaged cultures showed a significant increase (p<0.001) in proliferation relative to their controls at both D1 (~3-fold) and D3 (~5-fold). By contrast, in CBX-supplemented gentamicin-treated cultures there was no significant increase in proliferating cells at D1 relative to their control cultures and the already noted decrease at D3. For both the GZA- and CBX-supplemented cultures, the number of BrdU-labelled cells was lower at D3 compared with that at D1.

**Basilar papilla.** As with the utricular macula, GZA-supplemented gentamicin-damaged cultures showed a significant increase (p<0.01) in proliferation relative to their undamaged controls at D1 (Fig. IV.12B), while the increase in CBX-supplemented cultures was pronounced but not significant. No difference in the number of BrdU-labelled cells was observed in undamaged basilar papilla explants of 3d GZA- and CBX-supplemented cultures. However, there was a marked difference between GZA- and CBX-supplemented explants in the number of BrdU-labelled cells at both D1 and D3. In both culture systems a drop in the number of proliferating cells between D1 and D3 was observed.

![Figure IV.12. Comparison of BrdU-labelled cells in GZA- and CBX- supplemented explants.](image)

Data are expressed as the mean number of BrdU-labelled cells per mm² (± SD) for control and gentamicin-treated explants of (A) utricular macula and (B) basilar papilla. (*) indicates significant differences between GZA- and CBX- supplemented explants (p<0.05; one-way ANOVA).
3. Discussion: Methodology

3.1. Organotypic cultures

The suitability of organotypic cultures in the study of hair cell regeneration following aminoglycoside-induced hair cell loss has been established for the basilar papilla (Oesterle et al., 1993; Frenz et al., 1998) and the utricular macula (Matsui et al., 2000) of the chicken. Organotypic cultures have been used to characterise proliferation in response to hair cell death and identify hair cell progenitors (Warchol and Corwin, 1996), to monitor hair cell differentiation (Stone et al., 1996), and to identify putative triggers for hair cell regeneration (Warchol et al., 1993; Tsue et al., 1994; Navaratnam et al., 1996; Oesterle et al., 1997; Oesterle et al., 2000; Bermingham-McDonogh et al., 2001; Witte et al., 2001; Warchol, 2002).

Morphological and functional studies demonstrate that the majority of organotypic cultures used in these present experiments survive in good condition for up to nine days. Connexin expression resembles the in vivo pattern and the intercellular communication between supporting cells is functional in organotypic cultures. Consistent with the mitotic arrest of cells in the postembryonic basilar papilla, only occasional BrdU-labelled nuclei have been observed in control basilar papilla explants, whereas the ongoing proliferation that occurs in vivo utricular maculae is maintained in vitro. The elevated number of cells in S-phase compared with those reported for 7-10 day old chickens (~125/mm² (Matsui et al., 2000)) is in accordance with the higher production rate of hair cells at E21 (Goodyear et al., 1999).

The present study demonstrates that gentamicin-induced hair cell loss is followed by an increase in proliferation of progenitor cells within the auditory and vestibular sensory epithelia. The 3-5-fold increase in DNA synthesis in the utricular macula relative to controls within 48 hours of hair cell death is in accordance with previous studies (Oesterle et al., 1993; Matsui et al., 2000). The amount of BrdU-labelled cells at the peak of proliferation in the basilar papilla is higher than that found in other in vitro studies (~250/mm² (Stone et al., 1996)), but lower than the proliferation in vivo (~875/mm² (Stone et al., 1999)) following gentamicin induced hair cell loss. The temporal and spatial pattern of DNA synthesis in vivo mirrors the progression of hair cell loss (Hashino and Salvi, 1993; Hashino et al., 1995). In contrast, in basilar papilla explants, BrdU-labelled cells were, despite complete hair cell loss, confined to the neural edge and proliferation progressed distally to proximally. The extent and the faster timescale of hair cell death may contribute to these differences in proliferation patterns.
Despite the confinement of BrdU-labelled cells to the neural edge, newly regenerated hair cells were visible across the basilar papilla. This and the absence of surviving or sublethally damaged hair cells strongly suggests that a substantial population of supporting cells in the explants convert directly into hair cells. Consistent with the hair cell differentiation observed in the basilar papilla in vivo, immature hair cells became visible 3 days after the onset of aminoglycoside-damage. The newly formed hair cells matured in a manner which is consistent with previous studies (Stone and Rubel, 2000) and which resembled embryogenesis (Cotanche, 1987).

3.2. Pharmacological blocking of gap junction

The present study demonstrates that the CBX-induced decrease of gap junctional communication in the basilar papilla and utricular macula has a negative impact on the mitotic activity of supporting cells in response to hair cell loss, but no significant effect on the ongoing proliferation in the intact utricular macula at E21.

Caution has to be taken in the interpretation of these data, considering that the CBX-induced closure of gap junction channels is incomplete and accompanied by side effects, of which some may be cytotoxic. The quantification shows high variations in numbers of BrdU-labelled cells, which might have been caused by differences in the degree of hair cell loss, cell viability and/or efficiency of the CBX-block. The decrease of mitotic activity in GZA-supplemented control utricular maculae over time might be caused by initial stimulation of proliferation provoked by the attachment of the explants and/or the inhibition of proliferation resulting from the deterioration of supporting cells. Morphological and functional data indicate that CBX had no obvious adverse effects on supporting cells, but caused hair cell death during prolonged incubation periods. The increase in the mitotic activity observed in control cultures incubated in CBX for 5 days are thus likely to have been directly caused by increased hair cell death.

Furthermore, the differences in the level of proliferation between GZA- and CBX-supplemented cultures in response to gentamicin-induced hair cell loss might not be a secondary effect of the disruption of intercellular communication, but be caused directly by CBX. It has been suggested that the inhibition of the DNA synthesis of hepatic stellate cells by CBX is caused by the direct interference with the MAPK pathway rather than the blockages of gap junctional communication (Uyama et al., 2003). In addition, glycyrrhetinic acid has been reported to inhibit cell proliferation of pituitary tumour cells through its inhibitory effect on 11β-hydroxysteroid dehydrogenases, which prevents the conversion of cortisol to cortisone (Rabbitt et al., 2003).
V. DISCUSSION

1. Gap Junctions in the Avian Inner Ear

The combination of FRAP and immunohistochemistry revealed the following major characteristics of gap junctions in the avian inner ear:

1) Supporting cells in the basilar papilla and the utricular macula form functional syncytia from which hair cells are likely to be isolated;
2) cCx31 is a major connexin isoform in both the cochlear duct and utricle of hatchling chicks, where it shows a distinctive distribution within supporting cells;
3) Cx43 is expressed in the supporting cells of the basilar papilla, where it co-localises with cCx31, but is absent in the vestibular sensory epithelium;
4) Calcein spreads asymmetrically across the basilar papilla but uniformly in the utricular macula.

Connexin expression in the avian inner ear

cCx31, detected by the co-localisation of antibodies to the mammalian Cx26 and Cx30, is abundantly expressed in the cochlear duct and utricle of hatchling chicks and is likely to constitute the main connexin isoform in the avian inner ear. In the cochlear duct, cCx31-like immunostaining is present in the supporting and border cells of the sensory epithelium, in the clear cells along the neural edge and in the cuboidal and hyaline cells along the abneural edge of the basilar papilla, in the homogene cells of the lateral wall, and in the tegmentum vasculosum. In the utricle, cCx31-like gap junction plaques are confined to the supporting cells of the sensory epithelium and the cells of the transitional zone.

The expression pattern of cCx31 protein is generally consistent with the detection of its messenger RNA (mRNA). In 14-19 day-old chick embryos, cCx31 mRNA has been detected by in situ hybridisation in supporting cells, clear cells, cuboidal cells and in the tegmentum vasculosum (Heller et al., 1998). However, little to no cCx31 mRNA was reported in homogene and hyaline cells. The differences between immunohistochemistry and in situ hybridisation may be due to developmental variations between chick embryos and hatchlings or the cross-reactivity of the antibodies with an additional connexin protein.

The distribution pattern of cCx31 gap junctions is comparable to that of Cx26/Cx30 channels in the mammalian inner ear. Cx26 and Cx30 gap junctions join the supporting cells of the sensory epithelia and the cells comprising the connective tissue of the inner
ear, with the notable exception of cochlear and vestibular hair cells, the marginal cells of the stria vascularis and the vestibular dark cells (Kikuchi et al., 1994; Kikuchi et al., 1995; Forge et al., 2003a), to form syncytia.

In contrast to cCx31 gap junctions, the expression pattern of Cx43 in the avian inner ear is opposite to that of its orthologue in the murine inner ear. Cx43-positive gap junctions have been detected by immunohistochemistry between supporting cells in the basilar papilla of chicks, but not between those in the organ of Corti of mice. In addition, Cx43 is not detectable in gap junction plaques between supporting cells in the avian utricular macula, but is present between those in the utricular macula of mice (Forge et al., 2003a). Western blot analysis revealed that Cx43 is predominantly phosphorylated in the inner ear of chick hatchlings. Two phosphorylated forms, P1 and P2, were detected in homogenates of both the cochlear duct and utricle.

The distribution pattern of immunodetectable gap junctions in the inner ear of chick hatchlings is consistent with ultrastructural studies of the avian cochlear duct, in which gap junctions were identified between supporting cells (Forge et al., 2003a), between adjacent border cells, between border cells and supporting cells, between border and hyaline cells (Oesterle et al., 1992), and between the light cells of the tegmentum vasculosum (Hossler et al., 2002). No gap junctions could be detected between supporting cells and hair cells in the auditory and vestibular epithelia using freeze fracture (Forge et al., 2003a) and the absence of calcein transfer between hair and supporting cells lends support to the proposed functional isolation of avian hair cells. Such isolation of hair cells from the syncytium of the supporting cells is to be expected as each hair cell relies on finely adjusted potassium currents that underlie its mechanotransduction and electrical tuning. However it should be noted that gap junctions between supporting cells and hair cells have been identified in the vestibular end organs of the alligator lizard (Mulroy et al., 1993) and the goldfish (Hama 1980). To completely rule out coupling between the sensory and non-sensory cells, either the microinjection of Neurobiotin – a small dye known to pass through the majority of connexin channels – or, preferably, the measurement of electrotonic coupling by double patch clamp, has to be undertaken. As neither ultrastructural nor functional studies of the avian dark cells have been conducted yet, their functional isolation remains speculative. However, such an absence of immunodetectable gap junctions in the dark cell region of the avian utricle mirrors the known absence of gap junctions in marginal and dark cells of the mammalian inner ear.
**cCx31: A chimera of Cx26 and Cx30**

It is intriguing that cCx31, which is uniquely expressed in the inner ear (Heller et al., 1998), can be considered almost a chimera of Cx26 and Cx30, the predominant isoforms in the mammalian inner ear. As Cx26 and Cx30 are co-localised within the same gap junction plaque in the mammalian inner ear (Ahmad et al., 2003; Forge et al., 2003a), the physiological properties of cCx31 and Cx26/Cx30 channels may thus be crucial to the functioning of the vertebrate inner ear.

Comparative electrophysiological and dye-transfer studies of HeLa cells expressing cCx31 or co-expressing Cx26/Cx30 need to be made to illuminate common channel properties. Similarities are more likely to be found in the electrophysiological properties, especially in voltage gating, rather than in the permeability of the gap junctions. Dye-transfer studies of a chimeric Cx26/Cx30 construct, resembling the sequence of cCx31 (i.e. the N-terminal, transmembrane domains, extracellular loops and cytoplasmic loop have a Cx26 sequence while the C-terminal has a Cx30 sequence), point to different permeabilities for larger molecules. HeLa cells co-transfected with Cx26 and Cx30 (Marziano et al., 2003) did not transfer the large anionic dye Lucifer yellow to neighbouring cells while HeLa cells expressing this chimeric construct did (Manthey et al., 2001).

Another property of Cx26/Cx30 channels is their electrically and chemically (Forge et al., 2003b) rectifying junctions, which could result in an asymmetric current flow between the supporting cells (Zhao, 2000; Zhao and Santos-Sacchi, 2000). However, under the assumption that cCx31 is the major connexin isoform in the utricular macula, homotypic cCx31 junctions do not produce “rectifying” junctions. Diffusion patterns of calcein between supporting cells point to a uniform coupling of supporting cells within the utricular macula. But in the basilar papilla, calcein diffuses asymmetrically between supporting cells, with a predominant direction from the abneural to the neural edge.

**Cx43 and asymmetric dye transfer**

The notable difference between the auditory and vestibular system of the chicken is the expression pattern for Cx43. Cx43 is present between supporting cells of the basilar papilla, though not in those of the utricular macula, where Cx43 co-localises with cCx31. Furthermore, the downregulation of Cx43 following gentamicin-induced hair cell loss coincides with the loss of asymmetric calcein transfer. The formation of cCx31/Cx43 junctions is therefore likely to be implicated in the creation of directional pathways for calcein in the inner ear.
Asymmetric coupling has been observed so far between Müller glial cells and astrocytes in the retina (Robinson et al., 1993; Zahs and Newman, 1997) and between astrocytes and oligodendrocytes in the central nervous system (Robinson et al., 1993; Zahs, 1998). While these cells are heterologous, supporting cells are regarded as a homologous cell population. Thus, this may be the first evidence for homologous asymmetric coupling through gap junctions. Alternatively, epithelial supporting cells are not as homologous as previously thought. Though morphologically similar, differences manifest themselves in molecular gradients along and across the basilar papilla. The supporting cells at the neural edge are a striking example for such a gradient as they not only lack Cx43, but also FGFR3 (Bermingham-McDonogh et al., 2001) and calbindin (Hiel et al., 2002).

Detailed ultrastructural studies in combination with immunogold-labelling must now to be undertaken in order to uncover the precise arrangement and composition of gap junctions underlying this asymmetric dye transfer of calcein. Double immunofluorescence revealed that Cx43 is concentrated on one side of the junction, which is indicative of heterotypic junctions, or segregated in distinct domains within cCx31-like plaques. As the sequence motifs in cCx31 responsible for the determination of compatibility of connexins and connexons are more similar to those of Cx26 than Cx30, it is likely that cCx31 and Cx26 also show comparable arrangements within gap junction channels and plaques. Although Cx26, Cx30 and Cx43 have been reported to be present in the same plaque in astrocytes (Nagy et al., 2001), Cx43 and Cx26 form neither heterotypic (Manthey et al., 2001) nor heteromeric junctions, but segregate in distinct domains within a plaque (Gemel et al., 2004). By contrast, Cx43 and Cx30, despite their membership of different connexin classes, are able to form heterotypic, and possibly heteromeric, junctions (Altevogt and Paul, 2004).

**Functional implications of gap junctions in the avian inner ear**

Gap junctional communication among glia cells has been implicated in the clearance of extracellular $K^+$ within the central nervous system to prevent the interference of excess potassium with transmitter release and the electrical properties of the axons. It is assumed that one mechanism by which the ionic homeostasis is maintained involves the redistribution of $K^+$ from regions of high extracellular concentrations to regions with low extracellular concentrations (Orkand et al., 1966). This “spatial buffering” of $K^+$ is passive and energy-independent as it is driven by the local differences between the membrane potential and potassium equilibrium potential.
of the glial syncytium. The accumulation of extracellular potassium results in the increase of local $E_k$, which leads to an influx of potassium. The subsequent depolarisation of the glia membrane can spread electrotonically via gap junctions to more distal regions of the syncytium, where the $E_k$ is more negative than the membrane potential due to the tendency of the syncytium to remain isopotential. This leads to an outwardly directed force for potassium.

Slowly depolarising DC responses during sound stimulation in supporting cells of the guinea pig cochlea (Oesterle and Dallos, 1990) and the goldfish saccule suggest a similar involvement of supporting cells in the spatial buffering of potassium ions ($K^+$) in the vertebrate inner ear. Electrophysiological studies of supporting cells in the pigeon (Masetto and Correia, 1997) and the goldfish (Sugihara and Furukawa, 1996) support this hypothesis. The slightly inward rectifying $K^+$ current observed in supporting cells in the pigeon (Masetto and Correia, 1997) and the goldfish (Sugihara and Furukawa, 1996) is indicative of potassium channels that are open at resting potential and are further activated at very negative potentials. Due to the relatively large negative resting potential of vestibular supporting cells (-50 mV), their membrane should be preferably permeable for $K^+$ following the increase of extracellular $K^+$ and the subsequent shift in the local $E_k$. This provides the necessary driving force for $K^+$ to enter supporting cells. The inward rectifier improves the efficiency of spatial buffering by enhancing the $K^+$ influx in regions of elevated extracellular $K^+$ and by spreading the membrane depolarisation to more remote parts of the syncytium.

After their uptake, $K^+$ may be conveyed through the gap junction network of the supporting cells and released at some distance, where extracellular $K^+$ is lower. The expression pattern of Cx43 lends support to the involvement of gap junctions in the spatial buffering of $K^+$, in that the size and number of Cx43-positive junctions parallels the increase of outward $K^+$ currents from the distal to the proximal end of the basilar papilla (Pantelias et al., 2001). This gradient reflects the systematic variation in the number and kinetics of $K^+$ channels in the basolateral membrane of hair cells, which underlies their electrical tuning (Fettiplace and Fuchs, 1999). In accordance with the gradient of $K^+$ outward current, the level and pattern of Cx43 expression increases towards the proximal tip of the basilar papilla, where it is concentrated just below the basal surfaces of the tall hair cells overlying the neural limbus. These tall hair cells are considered the primary sensory receptors as they receive the majority of afferent innervation. Cx43-channels, which have a high unitary conductance (198 pS in the chicken) and are selective for cations (Veenstra et al., 1995), might provide an
additional pathway or modify the permeabilities of cCx31 channels, required for an efficient spatial buffering of K⁺ in high frequency regions of the basilar papilla.

Though asymmetry in dye transfer does not necessarily resemble the diffusion patterns of second messengers and even less of ions (Goldberg et al., 1999), it is tempting to speculate that there is a directional pathway in the basilar papilla which might underlie potassium recycling and/or signalling within the auditory epithelium. Gap junctions have been implicated in K⁺ cycling in both the mammalian cochlea and the vestibular system. Such potassium cycling maintains the high K⁺ concentration in the endolymph and participates in the generation of the endocochlear potential in the scala media. The endolymph in the avian inner ear has a similar K⁺ concentration (140-160 mM) (Ninoyu et al., 1987; Runhaar et al., 1991; Sauer et al., 1999) to that of the mammalian endolymph but, because of functional and structural differences between the tegmentum vasculosum and stria vascularis, the EP is only +20 mV (Necker, 1970; Cotanche et al., 1987) compared to +80 mV in the mammalian cochlea. Based on the similar morphology and EP, the mechanisms underlying the ion-transporting properties of dark cells in the avian inner ear are thought to be comparable to those of the mammalian vestibular dark cells. The entire avian gap junction system is therefore likely to resemble that of the mammalian vestibular system and to play a role mainly in potassium cycling.

In the avian cochlea, assuming that K⁺ follows the directional pathway visualised by calcein transfer, potassium would move predominantly across the basilar papilla, from the supporting cells of the abneural edge to the neural edge, through the clear cell and homogene network to the tegmentum vasculosum. Here it would be taken up by dark cells through Na⁺, K⁺- ATPase at their basolateral membrane folds (Schneider et al., 1987) and ejected into the endolymph. In the avian utricle, K⁺ might be transported either via the cCx31-gap junctions of the epithelial cells or via Cx43-gap junctions within the stroma and released into the extracellular space surrounding the dark cells.

Gap junctions might not only mediate potassium cycling but also intercellular signalling. The distinct distribution of gap junction plaques within the supporting cells may be the morphological manifestation of these different functions. A gap junction feature common to the inner ear of birds, reptiles and mammals is their distinctive distribution within supporting cells (Forge et al., 2003a). Plaque sizes within the supporting cell soma differ. Relatively small junctions are found towards the lumenal surface of supporting cells with much larger plaques towards the base of the cells. It has been suggested that there may be a functional correlation between the different plaque
sizes and differences in permeability (Forge et al., 2003a). Preliminary data of the dye spread within the supporting cells of the basilar papilla obtained during this study support this hypothesis, as the gap junctions at the lumenal surface had a higher permeability for calcein than the plaques at the basal surface. This difference in permeability may be a consequence of differences in the access of molecules as a result of the various plaque sizes and arrangements of channels within gap junctions (Hall and Gourdie, 1995), and/or a heterogeneity in connexin expression within supporting cells. Although the spread of calcein cannot be regarded as a true representation of the transfer of second messengers or ions, it is nevertheless tempting to speculate that two separate pathways with different functions join adjacent supporting cells, e.g. the basal gap junctions may be responsible for the uptake and spatial buffering of potassium ions, while lumenal gap junctions may mediate signalling between supporting cells.

2. Gap Junctions during Hair Cell Regeneration

The most prominent feature of gap junctions in response to gentamicin-induced hair cell death and the subsequent hair cell regeneration is the dramatic change in the Cx43 expression within the auditory sensory epithelium. Cx43 expression in the supporting cells began to decline around six hours after gentamicin addition and disappeared completely after 24 hours. This decrease is well before the entry of supporting cells into S-phase, but coincides with the death and extrusion of hair cells from the sensory epithelium and the closing of the lesion by the expansion of the supporting cell surfaces, during which the tight and adherens junctions between hair and supporting cells are rearranged and new intercellular junctions are formed (Forge, 1985; Cotanche and Dopyera, 1990; Raphael and Altschuler, 1991). After a further 24 hours, by which time the majority of supporting cells entered the S-phase, Cx43-immunostaining reappeared at the lumenal surface of the supporting cells.

The degradation of Cx43 and its regulation

Tight junctions and adherens junctions are known to be focal points of multi-protein complexes, which link a variety of transmembrane and signalling proteins with the cytoskeleton (Lapiere, 2000; Nagafuchi, 2001; Gonzalez-Mariscal et al., 2003; Perez-Moreno et al., 2003). There is increasing evidence that gap junctions and especially Cx43 are associated with these multi-protein complexes (Duffy et al., 2002; Herve et al., 2004). Cx43 has been shown to interact with tight junction proteins such as
occludin (Nagaoka et al., 1999) and ZO-1 (Giepmans and Moolenaar, 1998), and with the adherens junction proteins E- and N-cadherin, α- and β-catenin, and p120 (Suzuki et al., 2001; Xu et al., 2001). In addition, Cx43 can interact directly with the cytoskeleton via α- and β-tubulin (Giepmans et al., 2001). The temporal disruption of intercellular junctions and the rearrangement of the cytoskeleton after hair cell loss may thus have an impact on the Cx43 expression, as exemplified by the simultaneous downregulation of Cx43, claudins, occludin and VE-cadherin in the perineurium of sciatic nerves following the breakdown of the blood-nerve barrier (Hirakawa et al., 2003).

ZO-1, which belongs to the protein family of membrane associated guanylate kinases (MAGUK), is associated with tight junctions, where it links the transmembrane proteins to the actin cytoskeleton (Gonzalez-Mariscal et al., 2000). Amongst others, ZO-1 has been demonstrated to play a role in Cx43 turnover, as the disruption of intercellular contacts between myocytes (Barker et al., 2002) and Sertoli cells (Defamie et al., 2001) and the subsequent endocytosis of gap junctions was accompanied by an increased interaction between Cx43 and ZO-1. In support of ZO-1 as a stabiliser of gap junction plaques, Cx43 mutants that lack the ZO-1 binding domain exhibited an increased turnover rate (Toyofuku et al., 2001).

In addition, the removal of Cx43 from the plasma membrane may be associated with changes in the phosphorylation of the protein. Gap junctional communication mediated by Cx43-channels has been shown in a number of cell types to steadily decrease during their progression through the cell cycle, reaching a minimum at mitosis (M-phase) (Stein et al., 1992; Koo et al., 1997; Bittman and LoTurco, 1999; Solan et al., 2003). The decrease of Cx43 during G0/S is dependent on the phosphorylation by PKC (Koo et al., 1997), which has been implicated in the S-phase entry of supporting cells in the avian utricular macula (Witte et al., 2001).

**Cx43 and cell growth**

It has long been suggested that gap junctions have a negative influence on cell growth. The mounting evidence that tumour cells show aberrant gap junctional communication (Trosko and Ruch, 1998; Yamasaki et al., 1999) supports this hypothesis. Cx43 has been identified as a tumour suppressor gene, as it can restore normal cell growth in a variety of transformed and neoplastic cells (Zhu et al., 1991; Chen et al., 1995; Huang et al., 1998; Goldberg et al., 2000; Zhang et al., 2001). The mechanisms for this growth inhibition identified to date are varied and suggest a cell-specific regulation. Mechanisms both dependent on and independent of gap junctional
communication have been implicated in the regulation of cell proliferation by Cx43 (Krutovskikh et al., 2000; Moorby and Patel, 2001; Qin et al., 2002). In C6 glioma cells, Cx43 has been implicated in inducing contact growth inhibition by suppressing the extracellular milk fat globule epidermal growth factor 8 (MFG-E8) (Goldberg et al., 2000). In human glioblastoma cells, Cx43 transfection resulted in the reduction of the cytokine, monocyte chemoattractant protein 1 (MCP-1) (Huang et al., 2002). Increased levels of the CDK inhibitor p27 are thought to be responsible for the suppression of proliferation of osteosarcoma U2OS cells with enforced expression of Cx43 (Zhang et al., 2003). The increased synthesis combined with a reduced degradation of p27 has been shown to be dependent on increased cAMP. Alternatively, the involvement of Cx43 in the ubiquitin-mediated degradation of p27 may be through the direct modulation of S-phase kinase-associated protein (skp2), a mediator in the ubiquitination process. Furthermore, Cx43 has been shown to augment the suppression of the cyclin dependent kinase (cdk) inhibitor p21 by β-catenin/TCF-signalling (Kamei et al., 2003). These inhibitory effects of Cx43 on cell proliferation are consistent with its downregulation in the basilar papilla and its absence in the utricular macula, which has a constant turnover of hair cells.

It should be noted, however, that Cx43 has also been implicated in a positive role in proliferation. For example, a positive correlation with proliferation has been found during wound healing in the skin of mice (Coutinho et al., 2003) and in the development of the retina in the chicken (Becker and Mobbs, 1999). It is not surprising that such a ubiquitous connexin as Cx43 is subject to an array of regulatory mechanisms to modulate its properties according to cell type and physiology. This may account for the discrepancies in the findings that cAMP, which is known to increase trafficking and assembly of Cx43 (Atkinson et al., 1995; Burghardt et al., 1995; Paulson et al., 2000), has also been shown to increase proliferation in the undamaged and damaged basilar papilla (Navaratnam et al., 1996), and that Cx43 is not expressed in the mature organ of Corti (Forge et al., 2003a), which is unable to regenerate lost hair cells. One way of corroborating the involvement of Cx43 in hair cell regeneration in the avian inner ear is the specific knock down of Cx43 in the undamaged basilar papilla using antisense or siRNA technology and/or the forced expression of Cx43 via a retroviral vector in the utricular macula. The subsequent re-entry of supporting cells into the cell-cycle in the undamaged basilar papilla and the exit from the cell cycle of supporting cells in the utricular macula would conclusively prove the hypothesis that Cx43 gap junctions are involved in the inhibiton of supporting cell proliferation in the avian inner ear.
Cx43 gap junctions may not be the sole pathway along which signalling molecules are exchanged between supporting cells, as the non-specific blocking of gap junctions resulted in a decline of proliferation following hair cell loss. As discussed in chapter IV, the data gathered from the blocking experiments have to be interpreted cautiously, as CBX has been shown to suppress proliferation via its inhibition of 11β-hydroxysteroid dehydrogenase. Assuming that the blocking of gap junctional communication is the primary effect of CBX in the avian inner ear, it is likely that cCx31 gap junctions mediate the diffusion of a proliferation trigger, such as cAMP, and that the disruption of this pathway leads to a decrease in supporting cell proliferation. Alternatively, the disruption of general "housekeeping" might be enough to inhibit the re-entry of supporting cells into the cell cycle.

**Conclusion**

In summary, the main difference between the basilar papilla and the utricular macula is the differential expression of Cx43. Based on comparative functional and morphological studies, Cx43 may be implicated a) in the asymmetric spread of calcein, which may underlie a directional pathway in the inner ear for K⁺ cycling and/or intercellular signalling; and b) in the inhibition of supporting cell proliferation in the avian inner ear.
VI. References


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