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Functional Comparison of Connexin26 Wild-type and Non-syndromic Recessive Deafness Associated Mutant Channels

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Abstract of the Dissertation

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Gap junctions facilitate the exchange of ions, second messengers and small metabolites between adjacent cells. Gap junction channels are encoded by the connexin (Cx) gene family and show little selectivity to monovalent ions. However, gap junctions formed from different connexins are unique in terms of permselectivity to larger molecules. This phenomenon creates a problem in compensating for the loss of even one isoform in cases of disease causing mutations. Junctional communication plays an important role in cochlear homeostasis since mutations in the Cx26 gene (*GJB2*) are the leading cause of non-syndromic hereditary deafness. The exact function of intercellular communication in the inner ear is unknown. Two hypotheses were postulated, the first one focuses on the importance of potassium recirculation through gap junctions while the other emphasizes the role of biochemical coupling between cochlear supporting cells.

Partially active mutations would be an invaluable tool to differentiate the role of electrical coupling from metabolic coupling. Five Cx26 deafness mutants were screened in order to find channels that would retain some activity using a paired Xenopus oocyte expression system and dual whole-cell voltage clamp. Two of mutations (Thr8Met and Asn206Ser) formed active channels with properties different from wild-type Cx26. The single channel properties and permselectivity of wild-type and mutant Cx26 junctions were further characterized in transfected neuro-2A and HeLa cell pairs by dual whole-cell voltage clamp and intercellular fluorescent dye flux experiments. Protein expression and localization was verified by immunofluorescent staining. The average unitary conductance of wild-type and mutant channels was approximately 106pS in 120 mM potassium aspartate pipette solution, indicating comparable K^+ permeability. To look for possible differences in their permselectivity to larger molecules, the permeability of mutant and wild-type channels to Lucifer Yellow (LY) and ethidium bromide (EtBr) was analyzed in individual cell pairs. Mutant channels were as permeable to LY as wild-type Cx26 while the EtBr transfer through the mutant channels was greatly reduced compared to wild-type junctions. These findings corroborate with the hypothesis that the biochemical coupling through gap junctions is important for normal cochlear functioning and might also provide insight into the etiology of the hereditary hearing loss at the molecular level.

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List of Abbreviations

35delG- Deletion of Guanine at 30-35

42delE- Deletion of Glutamic Acid at 142

66delD- Deletion of Aspartic Acid at 167

167delT- Deletion of Thymine at 167

235delC- Deletion of Cytosine at 233-235

A40V- Mutation of Alanine at 40 into Valine

A88S- Mutation of Alanine at 88 into Serine

A88V- Mutation of Alanine at 88 into Valine

ADP- Adenosine diphosphate

AMP - Adenosine monophosphate (5'-adenylic acid)

ANOVA- Analysis of Variance

ATP- Adenosine 5'-triphosphate

BC- Basal cells

C86S- Mutation of Cysteine at 86 into Serine

cAMP- 3'-5'-Cyclic adenosine monophosphate

cDNA- Complementary Deoxyribonucleic acid

cGMP- Cyclic guanosine monophosphate

CMTX- X-linked Charcot-Marie Tooth disease

cRNA- Complemantary ribonucleic acid

Cx- Connexin

Cx26- Connexin 26

Cx30- Connexin 30

Cx30.3- Connexin 30.3

Cx31- Connexin 31

Cx31.9- Connexin 31.9

Cx40- Connexin 40

Cx43- Connexin 43

Cx45- Connexin 45

Cx46- Connexin 46

Cx50- Connexin 50

C-termini- Carboxyl termini

D50N- Mutation of Aspartic Acid at 50 into Glutamine

D66H- Mutation of Aspartic Acid at 66 into Histidine

DAPI- 4',6-Diamidino-2-phenylindole

DFNA3- Autosomal dominant non-syndromic sensorineural hearing loss

DFNB1- Autosomal recessive non-syndromic sensorineural hearing loss

EDTA- Ethylenedinitrilotetraacetic acid

EGTA- Ethylene glycol tetraacetic acid

EKV- Erythrokeratodermia variabilis

EP- Endolymphatic potential

ER- Endoplasmic reticulum

EtBr- Ethidium bromide

F83L- Mutation of Phenylalanine at 83 into Leucine

F137L- Mutation of Phenylalanine at 137 into Leucine

FC- Fibrocyte

G11R- Mutation of Glycine at 11 into Arginine

G12D- Mutation of Glycine at 12 into Aspartic Acid

G12R- Mutation of Glycine at 12 into Arginine

G12V- Mutation of Glycine at 12 into Valine

G45E- Mutation of Glycine at 45 into Glutamic Acid

G59A- Mutation of Glutamate at 59 into Arginine

GFP- Green fluorescent protein

GJA3- Gap junction alpha 3

GJB2- Gap junction beta 2

G_{jmax} -Maximum junctional conductance

G_{jmin}-Minimum junctional conductance

G_{jss}- Steady-state junctional conductance

HC- Hair cells

HED- Hidrotic Ectodermal Dysplasia

HEPES- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

I82M- Mutation of Isoleucine at 82 into Methionine

IC- Intermediate cells

IHC- Inner hair cell

I_j- Junctional current

I_{jss}- Steady-state junctional current

Inx- Innexin

IP_3- Inositol 1,4,5-trisphosphate

IRES- internal ribosome entry site

kDa- kilo Dalton

KID- Keratitis-Ichthyosis-Deafness syndrome

KO- knock-out

L34P- Mutation of Leucine at 34 into Proline

L90P- Mutation of Leucine at 90 into Proline

L214P- Mutation of Leucine at 214 into Proline

LY- Lucifer Yellow

M1V- Mutation of Methionine at 1 into Valine

M34T- Mutation of Methionine at 34 into Threonine

MB- Modified Barth's medium

MC- Marginal cells

mV- milli Volt

MW- Molecular weight

N206S- Mutation of Asparagine at 206 into Serine

NAD^+- Nicotinamide adenine dinucleotide

Neuro-2A- Mouse neuroblastoma cell line

nS- nano Siemens

N-termini- Amino termini

ODDD- Oculo-dento-digital dysplasia

OHC- Outer hair cell

OMIM- Online Mendelian Inheritance in Man

P173R- Mutation of Proline at 173 into Arginine

Panx- Pannexin

PPK- Palmoplantar keratoderma

pS- pico Siemens

R42P- Mutation of Arginine at 42 into Proline

R75W- Mutation of Arginine at 75 into Trp

R127H- Mutation of Arginine at 127 into Histidine

R143W- Mutation of Arginine at 143 into Tryptophan

R184P- Mutation of Arginine at 184 into Proline

RC- Root cells

S17F- Mutation of Serine at 17 into Phenylalanine

S19T- Mutation of Serine at 19 into Threonine

SC- Supporting cells

S.E. – Standard Error

siRNA- small interference ribonucleic acid

SNHL- Autosomal recessive non-syndromic sensorineural hearing loss

T8M- Mutation of Threonine at 8 into Methionine

V37I- Mutation of Valine at 37 into Isoleucine

V84L- Mutation of Valine at 84 into Leucine

V95M- Mutation of Valine at 95 into Methionine

V153I- Mutation of Valine at 153 into Isoleucine

V_0- Transjunctional voltage required to yield a conductance midway between maximum (G_{jmax}) and minimum conductance (G_{jmin})

V_j- Transjunctional voltage

W44S- Mutation of Tryptophan at 44 into Serine

W77R- Mutation of Tryptophan at 77 into Arginine

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Chapter I: Introduction

Gap junctional communication

Cellular communication is important for the maintenance of tissue/organ homeostasis in multicellular organisms. Using this communication, cells can review differences in environmental conditions and respond accordingly. This concept could involve either sending a signal to neighboring cells to generate a coordinated response or isolating groups of cells from the rest of the community to maintain tissue integrity. One type of communication between cells is mediated via intercellular channels which cluster in specialized regions of the plasma membrane to form gap junctions (Robertson, 1963;Revel and Karnovsky, 1967;Wei et al., 2004). Gap junctional channels link the cytoplasm of two cells, and provide a means for the exchange of ions (K^+, Ca^{2+}), second messengers (cAMP, cGMP, IP_3) and small metabolites (glucose), allowing electrical and biochemical coupling between cells (Figure I-1A) (Kanno and Loewenstein, 1964;Lawrence et al., 1978). Furthermore, Valiunas et al., recently showed that transfer of small interference RNAs between adjacent cells through gap junctions was possible; although it remains unclear if siRNAs are normally exchanged *in vivo* (Valiunas et al., 2005). Gap junctional communication is essential for many physiological events including cell synchronization, differentiation, cell growth and metabolic coordination of avascular organs such as epidermis and lens (Vinken et al., 2006;White and Paul, 1999).

Gap junctions are present in both vertebrates and invertebrates from mesozoa to mammals, while higher plants use structures called plasmodesmata for direct intercellular communication. In chordate animals, gap junction channels are encoded by a family of genes called connexins (Cx) (Goodenough, 1974), which can be categorized into five groups known as A, B, C, D and E according to their gene structure, overall gene homology and specific sequence motifs (Sohl and Willecke, 2003;Cruciani and Mikalsen, 2005)(http://www.genenames.org/genefamily/gj.php). There are two conventions of nomenclature in literature for connexin proteins, one of which depends on molecular mass of the connexin (Cx26 represents the connexin protein of 26kD; Cx46, connexin isoform of 46kD, etc.) while the other uses Greek symbols based on evolutionary considerations (*GJB2* is gap junction beta 2 referring to Cx26 while *GJA3* stands for gap junction alpha 3, or Cx46). Gap junctional communication in non-chordate animals, on the other hand, is mediated via another family of integral membrane proteins called innexins (Inxs). Innexin proteins are not homologous to connexins in terms of primary sequence, nevertheless gap junction channels formed from innexins share functional and structural similarities with intercellular channels made of connexins. Recently, another group of proteins called pannexins (Panxs) which may be distantly related to innexins were identified in vertebrates and have been shown to be expressed in various tissues including kidney, eye and neurons (Barbe et al., 2006;Panchin, 2005). Thus far, only mutations in connexin genes have been linked to human diseases.

Basic Structural Organization of Connexins and Gap Junctions

Gap junctions are highly specialized membrane structures that contain clusters of channels. This organization requires the membranes of two neighboring cells to come close to each other leaving a 2-4 nm gap (Bruzzone et al., 1996;White and Paul, 1999). Connexin family members share a similar structural topology (Figure I-1B). Each connexin has four transmembrane domains that constitute the wall/pore of the channels. These domains are connected by two extracellular loops that play a role in the cell-cell recognition and docking processes. There are three unchanged cysteine residues in each loop which solely form intra-connexin disulfide bonds. These cysteine residues are essential for the channel function as individual mutations of each cysteine failed to form functional channels (Dahl et al., 1992;Krutovskikh and Yamasaki, 2000). The transmembrane domains and the extracellular loops are highly conserved among the family members. Furthermore, connexin proteins have cytoplasmic N- and C- termini and a cytoplasmic loop linking the second and third transmembrane domains (Figure I-1B) (Lefebvre and Van De Water, 2000;Marziano et al., 2003). While the N-terminus is conserved, the cytoplasmic loop and C-terminus show great variation in terms of sequence and length. For example, the Cx26 protein has the shortest C-terminus while Cx50 has a long C-terminal tail. The cytoplasmic tail and loop are susceptible to various post-translational modifications (e.g. phosphorylation) which are believed to have regulatory roles (Cruciani and Mikalsen, 2002). Most connexins are phosphoproteins, and phosphorylation is thought to be important for the regulation of assembly and modulation of the physiological properties of the channels (Lampe and Lau, 2004;King et al., 2005).

Gap junction biosynthesis and assembly are strictly regulated and intercellular junctions have a short half-life of only a few hours (Musil et al., 2000). Most connexins are co-translationally integrated into the ER membrane. The oligomerization of six connexins into a hemichannel is thought to occur in a progressive fashion starting in the ER and ending in the trans-Golgi network (Sarma et al., 2002;Musil and Goodenough, 1993;Laird, 2006). Connexons (hemichannels) are then carried to the cell surface via vesicles transported through microtubules, which fuse to the plasma membrane. These hemichannels can either form non-junctional channels in unopposed areas of the cell membrane (see below) or diffuse freely to regions of cell-to-cell contact to find a partner connexon from a neighboring cell to complete the formation of intercellular channels (Figure I-1C) (Harris, 2001). These channels then cluster into gap junction plaques, a highly dynamic event involving removal of old channels from the center of the plaque while adding new gap junction subunits to the periphery (Gaietta et al., 2002). The intercellular channels from the middle of the plaque are internalized into vesicular structures called annular junctions (Jordan et al., 2001), which either fuse with the lysosome for degradation by lysosomal enzymes or are targeted to the proteosomal pathway (Qin et al., 2003;Laing and Beyer, 1995;Musil et al., 2000). The continuous synthesis and degradation of connexins through these mechanisms may facilitate the quick adaptation of tissues to changing environmental conditions. Unopposed hemichannels can also be functional under certain conditions, including mechanical and ischemic stress. Under these circumstances, open hemichannels are thought to facilitate the release of a variety of factors such as ATP, glutamate, and NAD^+ into the extracellular space, generating different physiological responses (Clarke et al., 2006). It is

currently not known if active hemichannels become incorporated in gap junctions prior to degradation, or follow a distinct recycling pathway.

There are 21 connexin isoforms in the human genome and nearly all cells in the body express at least one type of these genes at some point during development and in the adult life. For instance, Cx26 is highly expressed in cochlea, liver, skin and placenta while Cx46 and Cx50 are exclusively found in the eye. Moreover, connexins show overlapping expression patterns where an individual cell can use more than one type of isoform. Cx26, Cx30, Cx30.3, Cx31, Cx43 and others, for example, are found in keratinocytes (Kretz et al., 2003;Wiszniewski et al., 2000), whereas cardiomyocytes use Cx31.9, Cx40, Cx43 and Cx45 for intercellular communication (Beyer et al., 1995;Bukauskas et al., 2006). Co-expression of multiple genes within a single cell, consequently, can both affect the composition of connexons and intercellular channels that are formed. Connexons can be formed either from a single type of connexin or from more than one type, leading to the formation of either homomeric or heteromeric hemichannels, respectively. Another complexity is observed during the formation of the fully functional channels. Homotypic channels are formed from either the same homomeric or the same heteromeric connexons, while heterotypic channels contain different homomeric or heteromeric hemichannels (Figure I-1C). The formation of these structures depends on the compatibility of connexins forming the channels, since not all connexins can interact with each other such as Cx26 was shown to form heteromeric and/or heterotypic channels with Cx30 and Cx32 while it cannot form functional hybrid junctions with Cx40 (Segretain and Falk, 2004). These complex interactions thus increase

the structural and functional diversity, allowing a vast array of possibilities in the type of molecules shared between cells.

Permselectivity of Gap Junctions

Early models of gap junctional communication described the channels as being non-specific passive pores that would freely allow the passage of any ions or metabolites smaller than 1.2 kD (Simpson et al., 1977). However, recent developments in the field highlighted the selective permeability (permselectivity) of gap junction channels, demonstrating that channels formed from different connexins are unique in terms of their conductance, gating and ability to transfer specific molecules (Goldberg et al., 2004) (Figure I-2). Furthermore, the association of diseases to specific connexins has also emphasized the uniqueness of each protein, since the loss of one isoform cannot be compensated for by the presence of other connexins in the same tissue or a cell type and leads to pathophysiological defects. Gap junctions mediate the transfer of ions and metabolites/second messengers between cells, allowing ionic and biochemical coupling, respectively. In excitable cells such as neurons and heart, electrical coupling enables the generation of synchronized and rapid responses. In nonexcitable cells, the metabolic coupling may play role in the propagation of coordinated responses. To date, the ionic permeability of gap junctions made of different connexins has been shown to be similar to each other, presenting only minor alterations (Nicholson et al., 2000;Harris, 2001). On the other hand, diverse connexins demonstrated differences in the exchange of larger metabolites and second messengers (Nicholson et al., 2000;Weber et al., 2004;Goldberg

et al., 2004). This characteristic of connexins could explain why different tissues need different types of intercellular channels.

Bevans et al. were one of the first to demonstrate variation in the permeability of connexins to distinct signaling molecules (Bevans et al., 1998). They reconstituted liposomes with either Cx32 homomeric or Cx26/Cx32 heteromeric hemichannels and then loaded the liposomes with tritiated cAMP and cGMP. Using transport specific fractionation, they determined the permeabilities of these second messengers through the reconstituted channels and observed that Cx32 homomeric hemichannels were equally permeable to both cAMP and cGMP while the Cx26/Cx32 hybrid channels favored the passage of cGMP over cAMP (Figure I-2A).

In another study, Goldberg et al. compared the permeability of Cx32 and Cx43 to different cellular metabolites, including glutamate, glucose, adenosine, AMP, ADP, ATP and glutathione (Goldberg et al., 2002). They developed a layered culture system using a porous membrane to separate donor and receiver cells from each other, while still allowing the formation of gap junctions between these cells. This study demonstrated that Cx32 gap junctions had a 10-fold higher relative permeability to adenosine compared to Cx43 channels. On the other hand, the phosphorylation status of the adenosine shifted its preferential selectivity from Cx32 channels toward those formed from Cx43. The Cx43 intercellular channels were eight times more permeable to AMP and ADP than Cx32 channels, and the permeability of ATP through Cx43 was more than 400-fold better than that through Cx32 channels (Figure I-2B). These observations may suggest an effect of charge and size on the permselectivity of the molecule. However, Goldberg et al. had shown earlier that Cx32 intercellular channels were more permeable to calcein, a

fluorescent dye with higher molecular weight and charge (MW: 623 and charge: -4) than ATP (MW: 507 and charge: -3), relative to the Cx43 channels (Goldberg et al., 1999). These data suggest the presence of determinants of gap junctional permselectivity in addition to the size and the charge of the molecule.

The concept that each gap junction channel is unique in terms of permselectivity is supported by a large number of studies. This specificity is well demonstrated in connexin-associated diseases, where loss of one isoform cannot be compensated for by co-expressed connexins. Beltramello et al. compared the permeability of wild-type Cx26 and deafness associated Cx26 mutant V84L channels to IP_3 (Beltramello et al., 2005). Initially, they observed that both wild-type and mutant channels were equally permeable to K^+. Then, they extended their experiments to test if the passage of IP_3 between wild-type or mutant channels was altered. Although mutant V84L channels were permeable to potassium ions, the IP_3 transfer was impaired between the cells expressing the mutant protein. The authors also analyzed the permeability of Cx30 to IP_3 and determined that the permeability coefficient of Cx30 was approximately half that of wild-type Cx26. Hence, this suggests that even though Cx30 is highly homologous to Cx26, they are not functionally redundant and Cx30 may not be able to compensate for the loss of IP_3 exchange between the cochlear supporting cells in the absence of Cx26 (Beltramello et al., 2005). Furthermore, the passage of IP_3 and the generation of Ca^{2+} waves in organotypic cochlear cultures were examined to analyze if biochemical coupling through gap junctions was needed for normal cochlear function (Zhang et al., 2005). When IP_3 was injected into the wild-type cells, it caused the generation of Ca^{2+} waves in the cells adjacent to the injected one. However, the analysis of the deafness-causing mutants,

V84L, V95M and A88S, revealed a decrease in the intercellular transfer of a cationic fluorescent dye (propidium iodide) and IP_3 between cells. Moreover, these channels remained permeable to Na^+ and Ca^{2+} when the cells were directly injected with these ions and their diffusion was detected with fluorescent dyes (Zhang et al., 2005). This suggested that some of the deafness causing Cx26 mutant channels still retained their ionic coupling, but they had altered permeability to larger metabolites which may play a role in the etiology of the disease.

Physiological Importance of Gap Junctional Communication

Connexins play a key role in tissue/organ homeostasis and the etiology of several human hereditary diseases was linked to mutations in these genes. The first connexin related disease discovered in humans was X-linked Charcot-Marie Tooth disease (CMTX), which was due to mutations in the Cx32 gene (Bergoffen et al., 1993). Then, mutations in other connexins were found to be associated with a variety of human hereditary pathologies: Autosomal dominant cataract is caused by mutations in either Cx46 or Cx50 (Mackay et al., 1999;Shiels et al., 1998); oculodentodigital dysplasia is due to Cx43 mutations (Paznekas et al., 2003); and Cx31 or Cx30.3 mutations cause erythrokeratodermia variabilis (Macari et al., 2000;Richard et al., 1998a). Finally, dominant (DFNA) and recessive (DFNB) forms of nonsyndromic deafness are linked to mutations in three different connexin genes, Cx26, Cx30 and Cx31, which are widely expressed in the inner ear (Kelsell et al., 1997). In some cases, Cx32 mutations were found to cause deafness as well as CMTX (Stojkovic et al., 1999). Likewise, mutations in

Cx26 and Cx31 have been implicated in hereditary skin pathologies such as palmoplantar keratoderma and erythrokeratodermia variabilis as well as deafness, respectively (Table I-1) (Macari et al., 2000;Richard et al., 1998a). The linkage of connexin genes to human hereditary disorders implicated the crucial roles that gap junctional communication plays in the physiology of many tissues, including the eye, the cochlea and the skin. The characterization of disease causing mutations improves our understanding of the *in vivo* functions of these genes. Following sections will be focused on the effect of disease causing connexin mutations on channel biosynthesis and/or channel function first in the skin, and then in the inner ear, in order to elucidate the role of intercellular communication in these tissues.

Gap Junctional Communication in Skin

The epidermis is highly coupled by intercellular channels and gap junctional communication plays a crucial role in keratinocyte growth and differentiation. At least nine connexin genes, including Cx26, Cx30, Cx30.3, Cx31 and Cx43 were shown to be expressed during the keratinocyte differentiation process (Kelsell et al., 2000;Di et al., 2001). These genes show distinct spatial and temporal expression patterns as well as some overlapping tissue distribution during epidermal morphogenesis. Cx43 is expressed throughout the interfollicular epidermis while Cx26 is only present in palmoplantar epidermis. Cx26 and Cx43 are also expressed in the hair follicles and sweat glands (Salomon et al., 1994). Additionally, Cx30, Cx30.3 and Cx31 have been found in the upper, differentiated epidermal layers. Although the exact role of gap junctional

communication during keratinocyte differentiation is unknown, the association of mutations in the Cx26, Cx30, Cx30.3, Cx31, and Cx43 with different skin pathologies emphasized the importance of intercellular communication in the development and differentiation of epidermis (Richard, 2005). The first hereditary skin disease associated with connexin genes was erythrokeratodermia variabilis (EKV, OMIM# 133200), which is caused by mutations in the genes of either Cx31 or Cx30.3 (Macari et al., 2000;Richard et al., 1998a). In addition, mutations in Cx26, which are the leading cause of non-syndromic deafness, were also associated with a variety of skin disorders including palmoplantar keratoderma associated with sensorineural hearing loss (PPK; OMIM 148350) (Richard et al., 1998b), Keratitis-Ichthyosis-Deafness syndrome (KID; OMIM 148210) (van Steensel et al., 2002;Richard et al., 2002), Bart-Pumphrey syndrome (OMIM 149200) (Jan et al., 2004) and Vohwinkel syndrome (OMIM 124500) (Maestrini et al., 1999). Mutations in the Cx30 gene are the basis of Clouston Syndrome, which is also known as hidrotic ectodermal dysplasia (HED; OMIM 129500) (Lamartine et al., 2000). Finally, palmoplantar keratoderma, focal hyperkeratosis and kinky hair have been described in oculo-dento-digital dysplasia, which is a rare, complex developmental disorder mainly affecting the face, eyes, teeth, hair and limbs and is caused by mutations in the Cx43 gene (Kelly et al., 2006;Gong et al., 2006;Paznekas et al., 2003) (Table I-1). Here, the known changes in functional activity of mutant connexin proteins that produce the various skin disorders will be summarized.

Functional analyses of specific pathogenic connexin mutations associated with skin anomalies have been conducted to decipher the molecular mechanisms underlying these diseases and to highlight the normal role of intercellular communication in the

epidermis. In contrast to connexin mutations causing skin disorders, autosomal recessive hearing loss without skin involvement (non-syndromic) is often due to a simple loss of channel function altering cochlear intercellular communication (White, 2000;Bruzzone et al., 2003), suggesting that loss of connexin function is not detrimental for development and function of the epidermis. Thus, mutant connexin mutations proteins resulting in skin disorders must acquire novel functions not present in wild-type channels to generate epidermal abnormalities.

In *Xenopus* oocyte expression studies, three Cx26 mutants causing PPK and SNHL, R75W, delE42 and D66H, were found not only to lack gap junction channel function but also inhibited co-expressed wild-type Cx26 in a dominant-negative fashion, thus illustrating a unique molecular mechanism for the contribution of these mutations to skin disease (Rouan et al., 2001;Richard et al., 1998b). When these mutants were co-expressed with another epidermal connexin, Cx43, they also inhibited Cx43 channel activity. This suggested that PPK-associated Cx26 mutations might perform their action through a common mechanism where they act as trans-dominant inhibitors of other epidermal connexins, such as Cx43.

Analysis of the Cx26 mutations G59A and D66H in Vohwinkel syndrome provided further insight about the generation of distinct skin phenotypes (Thomas et al., 2004). Lucifer yellow dye transfer experiments demonstrated that these mutants failed to form functional channels. Co-expression with wild-type Cx26, Cx43 or Cx32 revealed that both G59A and D66H exerted a dominant-negative action on Cx26, while having selective trans-dominant effects on the other two connexins. G59A reduced dye transfer between cells when co-expressed with either Cx32 or Cx43. In contrast, D66H mutants

had a dominant-inhibitory effect only on Cx43 (Thomas et al., 2004). The differential ability of each mutation to affect distinct connexins may explain variations in epidermal phenotypes, or disease severity of specific mutations. Similar trans-dominant interactions were observed for other epidermal disease causing connexin genes. The EKV-associated Cx30.3 mutation F137L caused a reduction in the number and size of gap junctions formed, as well as decrease in coupling of HeLa cells when co-expressed with wild-type Cx31, suggesting a distinct trans-dominant inhibitory mechanism for this mutation (Plantard et al., 2003). Thus, the spectrum of trans-dominant interactions produced by distinct connexin mutations could uniquely alter the magnitude of intercellular communication and/or the range of permeable molecules shared between epidermal keratinocytes, leading to specific skin disorders such as PPK and EKV.

More evidence for the importance of dominant-negative action on co-expressed connexins came from the only animal model of a connexin skin disorder to date, mimicking Vohwinkel syndrome due to the expression of Cx26-D66H in transgenic mice (Bakirtzis et al., 2003). Cx26-D66H transgenic mice displayed premature keratinocyte cell death with a thickening of epidermal layers (hyperkeratosis), similar clinical features to human patients with heterozygous D66H mutations in *GJB*2 (Cx26). Accumulation of Cx26-D66H protein was observed in the cytoplasm of keratinocytes, implicating defective trafficking of the protein to the plasma membrane. Moreover, transgenic Cx26-D66H exerted a dominant negative effect on both Cx26 and Cx30, also inhibiting the transport of these wild-type proteins to the cell membrane. Surprisingly, Cx26-D66H did not interfere with Cx43 membrane localization (Bakirtzis et al., 2003) despite dominantly inhibiting Cx43 channel formation in two separate *in vitro* assays (Rouan et al.,

2001;Thomas et al., 2004). These results underscore the importance of animal models in confirming molecular mechanisms and suggest that additional research will be required to fully understand the pathogenicity of the D66H mutation.

EKV-associated Cx31 mutations were also shown to impair protein trafficking. The recessive L34P mutation caused protein accumulation in the cytoplasm and prevented the formation of functional Cx31 intercellular channels (Gottfried et al., 2002). Characterization of dominant Cx31 EKV-mutations such as R42P, 66delD, G12R, G12D and C86S further provided support for the role of impaired protein trafficking in this disease (Di et al., 2002). Mutant proteins showed an aberrant cytoplasmic localization and cells expressing mutant proteins had a high incidence of cell death. The defective trafficking and cell death were also detected in cells expressing Cx26 KID-associated mutants, G12R, S17F and D50N (Common et al., 2003). The reason for cell death has not been elucidated; however the defective proteins may influence the functionality of other connexins or other cellular components or alter the hemichannel function, thus leading to the observed skin pathologies.

In addition to impacting the function or assembly of gap junction channels, some connexin mutations may promote the activity of hemichannels. Support for this idea came from studies of a Cx26 KID syndrome mutation, A40V. Injection of A40V cRNA into *Xenopus* oocytes resulted in a disorganization of cell pigmentation followed by cell death. Moreover, induction of large membrane currents not seen in wild-type Cx26 suggested the presence of aberrant A40V hemichannel activity (Montgomery et al., 2004). Further support for the role of constitutivel active hemichannels in the etiology of KID syndrome came from the analysis of G45E mutation which was identified in two

infants with a rare fatal form of KIDS (Gilliam and Williams, 2002;Janecke et al., 2005). The expression of the mutant G45E cRNA in the *Xenopus* oocytes resulted in the pigment disorganization and blebbing of the ooplasm (Gerido et al., 2007). In addition, these oocytes displayed larger currents than either control or Cx26-injected cells in response to depolarization. The cell death and the large currents could be suppressed by the elevation of Ca^{2+} in the medium, indicating the presence of hemichannel activity. It was concluded that constitutively active hemichannels could contribute to the pathophysiology of these mutations in the generation of KIDS. Further support for disregulated hemichannels playing a role in pathology has emerged from studies of other skin disorders.

Similar to the A40V Cx26 mutation, the HED-associated Cx30 mutants G11R and A88V induced cell death in *Xenopus* oocytes, which could have resulted from the presence of functional hemichannels, an idea supported by the detection of large voltage-activated currents in single oocytes expressing mutant proteins that were not seen in cells injected with wild-type Cx30 (Essenfelder et al., 2004). These currents could be suppressed by elevation of extracellular calcium, and under these conditions, G11R and A88V formed functional intercellular channels with altered voltage gating properties. Further support for active hemichannels came from the analysis of ATP leakage in HeLa cells transfected with G11R and A88V. Cells expressing mutant channels had an ATP leakage 2-3 fold higher than control cells, suggesting that ATP release through hemichannels may play a role in the HED phenotype (Essenfelder et al., 2004). Aberrant channel activity was also observed for the EKV-associated Cx31 mutant G12R (Diestel et al., 2002). Cells expressing G12R started to die ~48 hours after initiation of protein synthesis. The mutant channels displayed increased neurobiotin permeability 24 hours

after protein expression relative to wild-type Cx31. This elevated junctional communication was proposed to be due to reduction or disruption of channel gating. However, these cells were not examined for functional hemichannels, which might have contributed to cell death. In addition to causing cell depolarization and death, hemichannels could induce the release of metabolites into the extracellular space in the epidermis and influence the regulation of keratinocyte growth and differentiation.

The etiology of several human hereditary skin diseases has been linked to mutations in a variety of connexin genes and different mutations within a single connexin gene can generate a range of functional defects, implicating that diverse channel activities contribute to the different disorders. Accumulating evidence shows that the mutations associated with distinct skin phenotypes give rise to novel functional activities. Three mechanisms could be proposed for the action of skin disease associated mutations: i) impaired protein trafficking and connexon assembly at the plasma membrane, ii) trans-dominant inhibition of co-expressed connexins and iii) the production of constitutively active hemichannels, any or all of which could potentially alter cell survival (Figure I-3). The trans-dominant inhibitory actions of mutant proteins change the range of channel types available in the epidermis, and therefore may alter the type of molecules exchanged and also the extent of intercellular communication. Leaky hemichannels might cause uncontrolled release of cell contents including metabolites and signaling molecules into the extracellular space which could affect the behavior of surrounding cells in addition to initiating cell death.

In the adult human epidermis, connexins have distinct as well as overlapping expression patterns. Under pathological conditions, the normal distribution of connexins

may be altered. For example, a PPK-patient with a Cx26-E42del mutation showed ectopic expression of Cx26 in the spinous and granular layers, where Cx26 was found to co-localize with Cx43 (Rouan et al., 2001). Co-localization of Cx26 with Cx43 in PPK lesions due to up-regulation of Cx26 could promote the interaction of Cx26 mutant protein with Cx43, altering its assembly and/or channel activity, consistent with *in vitro* studies showing a trans-dominant inhibition of Cx43 by Cx26 mutants (Rouan et al., 2001). The resulting alteration of gap junctional communication could modify the natural response pattern of keratinocytes during terminal differentiation and in response to stress or injury. It still remains unclear, however, how subtle changes in intercellular signal transmission within the upper layers of the epidermis result in epidermal thickening (acanthosis). Up-regulation of Cx26 in keratinocytes preceding hyper-proliferation has been observed during wound healing and inflammatory skin disorders (Djalilian et al., 2006;Labarthe et al., 1998). However, the relationship between the Cx26 up-regulation and keratinocyte proliferation is not fully known, and the signaling cascades and/or molecules that could play role in this process remain to be identified. Examination of skin samples from patients with other connexin based disorders, such as EKV or HED may further demonstrate if alterations in the connexin expression and distribution are a common feature of skin diseases. Additional characterization of disease causing mutations and the generation and examination of transgenic animals will further improve our understanding of the function of gap junctional communication in keratinocyte growth and differentiation as well as the pathophysiology of diseases where control of these processes has been corrupted.

Anatomy of the Inner Ear

The cochlea is the auditory portion of the inner ear. It contains the organ of Corti, the sensory organ of hearing which is composed of sensory hair cells, inner hair cells (IHC) and outer hair cells (OHC) and the non-sensory supporting cells (Figure I-4). The supporting cells include Deiters' cells, Hensen cells, Pilar cells and Claudius cells (Shim, 2006). The hairs do not express connexins, while the supporting cells are connected to each other through gap junction channels. In addition, there are no intercellular gap junction channels between hair cells and their supporting cells (Kikuchi et al., 1995). Another gap junction network provides connections between the fibrocytes of the spiral ligament and also the cells of the stria vascularis (Rabionet et al., 2002;Forge et al., 2002). The gap junction systems form a network that functionally couples the supporting cells in the inner ear and is thought to play an important role in the maintenance of cochlear ionic homeostasis (Kemperman et al., 2002;Forge et al., 2002;Kikuchi et al., 2000b;Wilcox et al., 2000).

There are two fluid filled compartments in the inner ear; the endolymph and perilymph. The basolateral surfaces of hair cells are immersed in the perilymph which has a ionic composition similar to other body fluids with high Na^+ and low K^+ concentrations (Forge et al., 2002). On the other hand, the apical sides of hair cells are embedded in the endolymph. The ionic composition of the endolymph resembles to that of cytoplasm, with high K^+ (150mM) and low Na^+ concentrations. Additionally, there is a positive 80-100mV potential in the endolymph relative to the perilymph, and the maintenance of the high potassium concentration and endolymphatic potential (EP) is

crucial for normal functioning of the cochlea. Upon sound stimulation, there is an influx of K^+ from endolymph into the hair cells. These ions are then released into the basolateral extracellular spaces between the hair cells and supporting cells. The surrounding supporting cells take up potassium ions through K^+/Cl^- co-transporters and recycle them back to the endolymph. Therefore, K^+ is removed from around the hair cells and returned back to the endolymph, maintaining the endolymphatic ionic composition and potential, as well as the sensitivity of hair cells to the next stimuli. The gap junctional network between supporting cells is suggested to play a role in the removal of K^+ ions around hair cells and their recirculation back to the endolymph after the initiation of auditory transduction (Forge et al., 2003;Kikuchi et al., 2000a;Zhao et al., 2006;White and Paul, 1999).

The Role of Intercellular Communication in the Cochlea

Gap junctional communication is important for cochlear homeostasis since mutations in different connexins are the leading causes of non-syndromic hereditary hearing loss (Van Hauwe et al., 1999;Marziano et al., 2003;Petit et al., 2001;Rabionet et al., 2002;Liu et al., 2001) (http://davinci.crg.es/deafness/). At least four connexins, Cx26, Cx30, Cx31 and Cx43 have been shown to be expressed in the cochlea (Kikuchi et al., 1994;Lautermann et al., 1998;Xia et al., 2000;Lautermann et al., 1999) . Cx26 and Cx30 are the predominant components of cochlear gap junction channels and immunocytochemical analysis demonstrated that Cx26 expression was correlated with the distribution of gap junctions in the inner ear (Lautermann et al., 1998;Jagger and

Forge, 2006;Kikuchi et al., 1994;Kikuchi et al., 2000a). Cx26 and Cx30 channels were found in the same gap junction plaques where they were shown to form hybrid channels (Lautermann et al., 1999;Forge et al., 2003;Marziano et al., 2003;Yum et al., 2007). Immunostaining of cochlear preparations revealed that Cx43 was mainly found outside of the organ of Corti with the exception of a few spots between the supporting cells. Cx43 was predominantly found in the stria vascularis and very weak staining of Cx43 was also detected between the fibrocytes of the spiral ligament and the spiral limbus (Lautermann et al., 1998;Suzuki et al., 2003). The cell specific expression and the distribution of connexins in the inner ear may suggest the necessity of diverse intercellular communication for cochlear homeostasis.

The exact function of gap junctional communication in the inner ear is not fully understood. However, it is widely accepted that the maintenance of potassium homeostasis is important for normal hearing processes and gap junctional communication is important for this to occur. Moreover, it has been suggested that the abnormalities in the cochlear intercellular communication due to connexin mutations may change the environment around the sensory hair cells thereby altering their survival and/or functionality (Cohen-Salmon et al., 2002). To date, two mechanisms have been proposed regarding the function of cochlear gap junctional communication in sustaining potassium homeostasis. The first model suggested that gap junctions facilitate the circulation of K^+ from around hair cells back to the endolymph, maintaining the endolymphatic potential and the sensitivity of hair cells for the next stimuli (Figure I-5). It was suggested that gap junction channels between the supporting cells provides a path for the movement of potassium from around the hair cells to the spiral ligament, and then the connective tissue

gap junction network transports them back to the endolymph in the stria vascularis (Kikuchi et al., 2000b;Wangemann, 2006). In cases of mutations in connexin diseases, the K^+ re-circulation mechanism fails, resulting in the accumulation of excess potassium around hair cells which then may lead to cell degeneration and/or death and eventually to hearing loss.

On the other hand, advancements in the field provided an alternative local mechanism for the role of gap junctional communication in the circulation of K^+ ions that involves the action of second messengers (Figure I-6). The removal of excess potassium around the hair cells by means of supporting cell gap junction system is crucial for cochlear homeostasis. Gap junction channels between cochlear supporting cells were also shown to be permeable to the second messenger IP_3 (Beltramello et al., 2005). It is suggested that IP_3 might be transferred between the cochlear supporting cells through the intercellular channels during removal of excess K^+ ions. The transfer of IP_3 between the supporting cells might result in the generation of Ca^{2+} waves, which is then speculated to activate a K^+/Cl^- efflux system that pumps the potassium ions back into the endolymph. Hence, not only the K^+ accumulation around hair cells is eliminated but also the endolymphatic potential is maintained (Bruzzone and Cohen-Salmon, 2005). Some connexin mutations might result in the alteration of biochemical coupling between the supporting cells even though they remained permeable to potassium (Beltramello et al., 2005). The alteration of IP_3 transfer between the supporting cells due to deafness mutations was proposed to inhibit the K^+ removal mechanism, leading to overloading of potassium ions around hair cells and eventually to deafness (Beltramello et al., 2005;Bruzzone and Cohen-Salmon, 2005).

Animal Models of Connexin Associated Hearing Loss

The characterization of animal models that have connexin dependent hearing loss phenotypes further established the importance and function of gap junctional communication in the cochlea. Cx26 knock-out mice were embryonic lethal, since loss of Cx26 in placenta resulted in the failure of glucose and other nutrients transport from maternal blood to the blood of fetus (Gabriel et al., 1998). Using a cochlear supporting cell specific promoter that was driven by Cre recombinase, the Cx26 coding region was specifically deleted from the cochlear epithelial cells in a conditional knockout model (Cohen-Salmon et al., 2002). The absence of Cx26 caused hearing loss in these animals. These animals had normal cochlear development until the onset of hearing, implicating that Cx26 dependent gap junctional communication was not essential for early stages of cochlear development. After the onset of hearing (P14), morphological examination of the inner ear revealed that the cells in the organ of Corti degenerated and underwent apoptotic cell death. Moreover, there was deformation of the organ of Corti and this was accompanied by a decrease in both the endolymphatic potential and potassium concentration in endolymph. It was speculated that the hair cells release both potassium and the neurotransmitter glutamate into the perilymph during hearing processes. The loss of Cx26 from the supporting cells might cause to the elevation of extracellular potassium concentration around hair cells, which might alter glutamate transport, leading to the accumulation of glutamate in the extracellular space. The glutamate overloading around the hair cells and their supporting cells could initiate apoptotic cell death. As mentioned before, Cx26 and Cx30 form heteromeric and/or heterotypic channels in the inner ear. To

see how Cx26 loss affects Cx30 in the cochlea, the comparison of the expression and distribution of Cx30 in the cochlea of conditional Cx26 KO and wild-type animals indicated that loss of Cx26 did not affect Cx30 tissue distribution (Cohen-Salmon et al., 2002).

The importance of Cx26 for normal hearing process was advanced by the generation of transgenic animals expressing deafness causing dominant negative mutants. The R75W mutation was unable to form functional channels in the paired *Xenopus* oocyte expression system and also inhibited the channel forming activity of wild-type Cx26 when co-expressed with this mutant channel (Richard et al., 1998b). The Cx26 R75W transgenic animals had normal development except for the presence of profound to severe hearing loss. The examination of cochlear morphology demonstrated that abnormalities in the supporting cells were apparent starting from 2 weeks after birth, and the degeneration and death of hair cells, especially OHCs, was evident in 7-week old animals. Moreover, the tunnel of Corti failed to form and the degeneration of the organ of Corti started to appear at 7-weeks. Opposite to the Cx26 conditional KO animals, there was no change in the EP of transgenic animals compared to wild-type mice. The characterization of the morphology and cells of the stria vascularis revealed that transgenic expression of Cx26 R75W mutant allele did not affect the organization and the survival of the cells in this region.

Cx30 is widely expressed in the inner ear and together with Cx26 and forms the main components of gap junction channels. The effect of Cx30 on auditory transduction and the physiology of the inner was analyzed using a Cx30 knockout mouse model. Cx30 loss did not interfere with the survival and development of these animals, it only caused

hearing loss. Cx30 knockout mice had normal cochlear and vestibular organ development until the onset of hearing. From this point on, abnormalities started to appear; the death of the IHC, OHC and their supporting cells was evident, which led to the disruption of the organ of Corti. Furthermore, the endolymphatic potential was completely abolished. Cx26 cellular expression and distribution was not affected by the absence of Cx30 channels (Teubner et al., 2003). Cx26 and Cx30 have the highest homology among members of the connexin gene family (74% amino acid identity) and homotypic channels made from either protein can efficiently pass K^+. Therefore, it was suggested that loss of Cx26 could be compensated for by the Cx30 in Cx26 conditional KO mice or vice versa in Cx30 KO and might be able to maintain the circulation of K^+ (Valiunas et al., 1999a;Kudo et al., 2003). However, animal models and deaf individuals with either Cx26 or Cx30 mutations suggested that this is not the case and that Cx26 and Cx30 are not functionally redundant in the inner ear.

Recently, it was suggested that the number of active channels, rather than presence of different channels types, might be important for the normal functioning of the cochlea. Further characterization of Cx30 KO animals provided support for this view (Ahmad et al., 2007). Even though it was shown that the Cx30 loss did not affect the tissue distribution of Cx26 in the cochlea, there was a significant reduction in the Cx26 cochlear protein level with no apparent change in the mRNA level compared to wild-type animals. The loss of Cx30 in the cochlea resulted in the decreased Cx26 protein and/or channel stability, which eventually led to reduction in the number of functional channels in Cx30 KO animals. It was suggested that overexpression of Cx26 to levels similar to wild-type animals in a Cx30 null background might reverse the defects observed in KO

animals. The transgenic restoration of Cx26 protein expression to wild-type levels in transgenic Cx30 KO animals resulted in animals with normal cochlear morphology and hearing. This suggested that significant overexpression of Cx26 was able to compensate for the loss of Cx30, implying that the number of active gap junction channels is crucial for normal hearing processes. Both Cx26 and Cx30 channels are known to permeable to K^+ ions however they show differences in their selectivity to larger molecules (Valiunas et al., 1999a;Manthey et al., 2001). Cx26 homomeric channels were permeable to both cationic and anionic molecules whereas Cx30 channels had preference for cationic molecules. Therefore, the absence of Cx30 might impair the exchange of cationic molecules between the cochlear supporting cells and this might be compensated for by the over expression of Cx26 in KO animals due to permeability of Cx26 to a broader range of molecules compared to Cx30.

Connexin26 Mutations in Hearing Loss

The linkage of the *GJB2* gene mutations to genetic deafness implicated the importance of gap junctions in the hearing process (Kelsell et al., 1997;Zelante et al., 1997). Mutations in Cx26 are associated with both syndromic and non-syndromic deafness. The genetic studies revealed that Cx26 mutations are the cause of up to 50% of inherited and 40% of sporadic nonsyndromic SNHL in diverse ethnic groups (Zelante et al., 1997;Cryns et al., 2004;Green et al., 1999). Recessive mutations in the *GJB2* gene are the most common cause of sporadic and autosomal recessive non-syndromic sensorineural hearing loss (SNHL). More than 50 distinct recessive mutations have been

described, including nonsense, missense, splicing, small insertions and deletions (http://davinci.crg.es/deafness/). The occurrence of some of these mutations has been shown to be predominant in certain populations. For example, the single nucleotide deletion 35delG accounts for more than 70% of the Cx26 associated hearing loss in individuals of Mediterranean (1 ⁄ 30- 1/ 35) and Northern European (1 ⁄ 50) origins (Gasparini et al., 2000;Cryns et al., 2004;Estivill et al., 1998;D'Andrea et al., 2002). On the other hand, the 167delT deletion is found to be prevalent in Ashkenazi-Jews (1 ⁄ 10) (Morell et al., 1998;Sobe et al., 1999) while the 235delC mutation is prevalent in Asian population such as Japanese, Korean and Chinese (1 ⁄ 100) (Kudo et al., 2001;Rabionet et al., 2000;Abe et al., 2000) .

Functional analysis of deafness associated mutations enabled the characterization of molecular mechanisms that play role in the generation of hearing loss and provided an understanding regarding the function of gap junctions in cochlea. Most of Cx26 mutations associated with non-syndromic recessive deafness generally resulted in loss of channel function. The mutations that lead to nonfunctional channels affect the channel biosynthesis at different levels from translation to the completion of the channel on the membrane, or lead to alterations in channel permeability. Some of the mutations were shown to interfere with the protein translation (Scott et al., 1998;Estivill et al., 1998;Wilcox et al., 1999;D'Andrea et al., 2002;Thonnissen et al., 2002). The deletion of one base (G) at positions between 30-35 nucleotides causes a frame shift in the 12th amino acid which results in the premature termination of protein translation. Moreover, the expression of the 35delG in HeLa cells were fail to produce protein (D'Andrea et al., 2002). This suggested that patients with homozygous 35delG mutation were completely

devoid of Cx26 protein and therefore Cx26 channels in their cochlea. Similarly, analysis of the Cx26 mutations M1V and P173R in mammalian cells revealed the inability of these variations to make protein (Thonnissen et al., 2002). Therefore, the complete loss of Cx26 channels from cochlea suggested alterations in the inner ear homeostasis which then results in the generation of hearing loss.

The second groups of mutations have been demonstrated to affect the oligomerization of connexins into hemichannels. Characterization of W77R and R184P mutations in mammalian cells indicated that these mutations completely disturbed connexon assembly, leading to the accumulation of mutant proteins within the cytoplasm. Consequently, they inhibited the formation of channels at the cell membrane so cells transfected with these mutations were not permeable to neurobiotin (Martin et al., 1999;Thonnissen et al., 2002). On the other hand, a subset of mutations was shown to partially abolish the formation of hemichannels (Oshima et al., 2003). The M34T mutation interfered with the oligomerization of Cx26 into hemichannels but a fraction of mutant channels was transported to the plasma membrane especially at the region of cell-to-cell contact. Further functional studies revealed that cells expressing this mutant protein were permeable to neurobiotin, implicating their ability to form functional channels (Thonnissen et al., 2002). Moreover, R75W and L90P mutations failed to assemble efficiently into hemichannels, but they could still be detected on the plasma membrane. However, they were unable to form active channels since cells transfected with the mutant DNAs had significantly reduced dye transfer between pairs.

Cx26 syndromic and non-syndromic mutations were also shown to acquire a dominant negative activity, where they inhibited the channel forming ability and/or the

permeability of wild-type Cx26 and/or other connexins such as Cx30 in co-expression assays (G59A, D66H, R75W, W44S, M34T, I82M and V37I) (Palmada et al., 2006;Kudo et al., 2003;Forge et al., 2003;D'Andrea et al., 2002). G59A and D66H mutant proteins were mainly intercellular and were not able to transfer Cascade blue when expressed in mammalian cells. Co-expression of each mutant channel with either wild-type Cx26 or Cx30 revealed that D66H mutants had a dominant negative effect on Cx26 while G59H interfered with the activity of Cx30 channels. Moreover, characterization of W44S and R75W revealed that they were able to form gap junction plaques. However, they demonstrated very little dye transfer and inhibited the ability of both wild-type Cx26 and Cx30 to form functional channels (Richard 1998 Cx26) (Forge et al., 2003;Marziano et al., 2003) . The characterization of M34T, I82M and V37I in *Xenopus* oocytes and in mammalian cells demonstrated the dominant action of these mutant proteins on wild-type Cx26 as well as on Cx30. M34T and V37 mutants resulted in the significant reduction currents recorded from the transfected cells (D'Andrea et al., 2002;Palmada et al., 2006).

The last group of mutations interfere with channel function and/or permeability (V84L, V95M, R143W and A88V) (Wang et al., 2003;Zhang et al., 2005;Beltramello et al., 2005;Bruzzone et al., 2003). These mutant channels have been demonstrated to assemble gap junction plaques on the plasma membrane when analyzed in mammalian cells. V84L, V95M and R143W formed gap junction channels with similar junctional conductance to wild-type Cx26 (Beltramello et al., 2005;Wang et al., 2003;Bruzzone et al., 2003). The channels were able to electrically couple cells. When tested in *Xenopus* oocytes or in mammalian cells, they were able to transfer simple ions and small molecules such as K^+, Na^+, Ca^{2+} and neurobiotin between the cells. However, the

permeability of these channels to larger molecules (IP_3, propidium iodide) was impaired. The characterization of mutations associated with deafness has provided an insight about both the role of gap junctional communication in the inner ear and also the etiology of hearing loss. These analyses had also improved the understanding of connexin and gap junction channel physiology, such as deciphering the role of amino acid residues and/or domains on the assembly of connexon/channel formation and channel properties, including channel docking processes, voltage gating properties, pH sensitivity and channel permeability.

Summary

Gap junctions facilitate both metabolic and electrical coupling between neighboring cells. The association of several connexins with a variety of human hereditary diseases implicates the importance of intercellular communication for tissue homeostasis. Gap junction channels formed from different connexins were initially believed to be non-selective for molecules smaller than 1.2 kDa. Therefore, loss of one isoform in the case of mutations could be potentially compensated for by the remaining intact connexins. Subsequently, it was shown that although connexins showed little selectivity to simple ions, each gap junction channel was selectively permeable to larger molecules.

Cx26, Cx30 and Cx31 are widely expressed in the cochlea and mutations in these genes are the leading causes of non-syndromic hearing loss, pointing out the significance of gap junctional communication in the inner ear. Cochlear intercellular communication was initially suggested to be crucial for the potassium ion re-circulation back to the endolymph

after the initiation of auditory transduction. However, a recently proposed mechanism pointed out an additional role of metabolic coupling in the removal of potassium ions around hair cells through gap junction networks. However, it remains unclear whether the electrical or metabolic coupling of cochlear supporting cells via intercellular channels is essential for normal cochlear functioning.

Hypothesis and Specific Aims

We suggest that the exchange of second messengers and/or small metabolites between the cochlear supporting cells by gap junction mediated intercellular communication is essential for normal hearing processes in addition to its suggested role in the potassium re-circulation pathway. The main hypothesis of this dissertation is that **the characterization of deafness associated Cx26 missense mutations that retain some level of functional activity would be an invaluable tool in order to distinguish the role of biochemical coupling from electrical coupling between the supporting cells through gap junctions.** This hypothesis was examined by the following two specific aims:

Specific Aim 1: Cx26 non-syndromic recessive deafness associated missense mutations will be screened in order to find mutant channels that retain some activity. The behavior of wild-type and selected mutant channels will be tested by using the paired *Xenopus* oocyte expression system and dual whole-cell voltage clamp. *Xenopus* oocytes will be injected individually with wild-type and mutant cRNAs. The oocytes will be paired and tested by dual-voltage clamp, which will enable the measurement of current passing through the intercellular

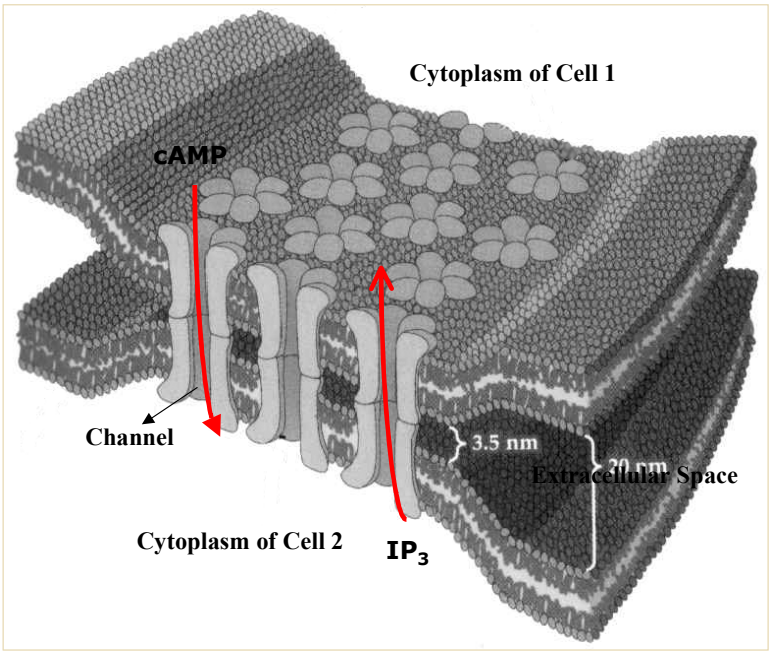
channels between the oocyte pairs. The comparison of junctional conductance between the wild-type and mutant channels will reveal the channel forming ability of mutant proteins. Characterization of channel properties might suggest some mechanisms about how the selected mutations could affect the channel function.

Specific Aim 2: The oocyte expression system provides a quick screen for the ability of mutant proteins to form channels, but gives information only about the macroscopic properties of channels. Partially functional mutations will be further characterized in transfected gap junctional communication deficient mammalian cell lines. The expression and localization of mutant proteins will be verified by immunofluorescent staining, followed by validation of the channel forming ability of mutant proteins in mammalian expression system. The permeability of mutant channels to K^+ will be compared with wild-type Cx26 by testing their single channel conductance properties using dual whole-cell voltage clamp in poorly coupled cells to analyze if the mutations interfere with potassium transfer between the cell pairs. Lastly, the permeability of partially active mutant channels to larger molecules will be analyzed using fluorescent dye flux experiments followed by dual whole-cell voltage clamp recordings. The differences between the mutant and the wild-type channels in terms of permeability to either potassium ions or larger molecules will emphasize the role of electrical or biochemical coupling for hearing processes, respectively.

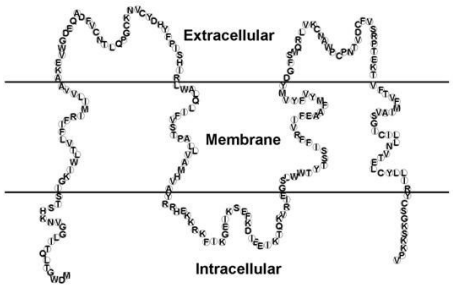
Figure Legends and Figures:

Figure I-1: Schematic representation of connexin and gap junction channels. A. Intercellular channels in the plasma membrane allow the exchange of ions, second messengers and small metabolites between adjacent cells (Evans and Martin, 2002). **B.** Connexins have four transmembrane domains, two extracellular loops, a cytoplasmic loop, and cytoplasmic amino- and carboxy-termini. **C.** Six connexins oligomerize to form hemichannels called connexons which then align in the extracellular space to complete the formation of gap junction channels. Different connexins can selectively interact with each other to form homomeric, heteromeric and heterotypic channels, which differ in their content and spatial arrangement of connexin subunits (Mese et al., 2007).

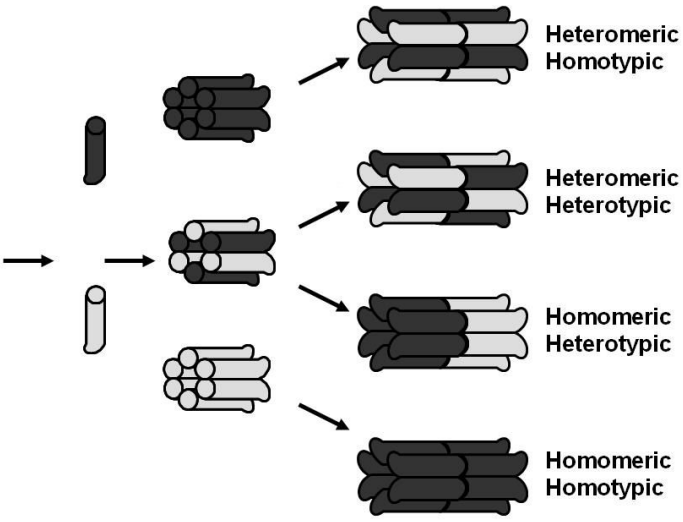
A



B



C



Connexin26 relative to the cell membrane Connexin Connexon Gap Junctions

Figure I-2. Selective permeability of gap junctions to second messengers. **A.** The composition of gap junction channel alters its permeation to large metabolites. Cx32 hemichannels were shown to permeable to both cAMP and cGMP while Cx26/Cx32 heteromeric hemichannels showed reduced transfer of cAMP but their permeability to cGMP was comparable to that of Cx32 hemichannels. **B.** The addition of extra phosphate groups to adenosine changed its relative permeability through gap junctions. Cx32 intercellular channels were more permeable to adenosine than channels formed by Cx43. However, the Cx43 intercellular channels had progressively higher permeability to AMP, ADP and ATP than those formed from Cx32 (Mese et al., 2007).

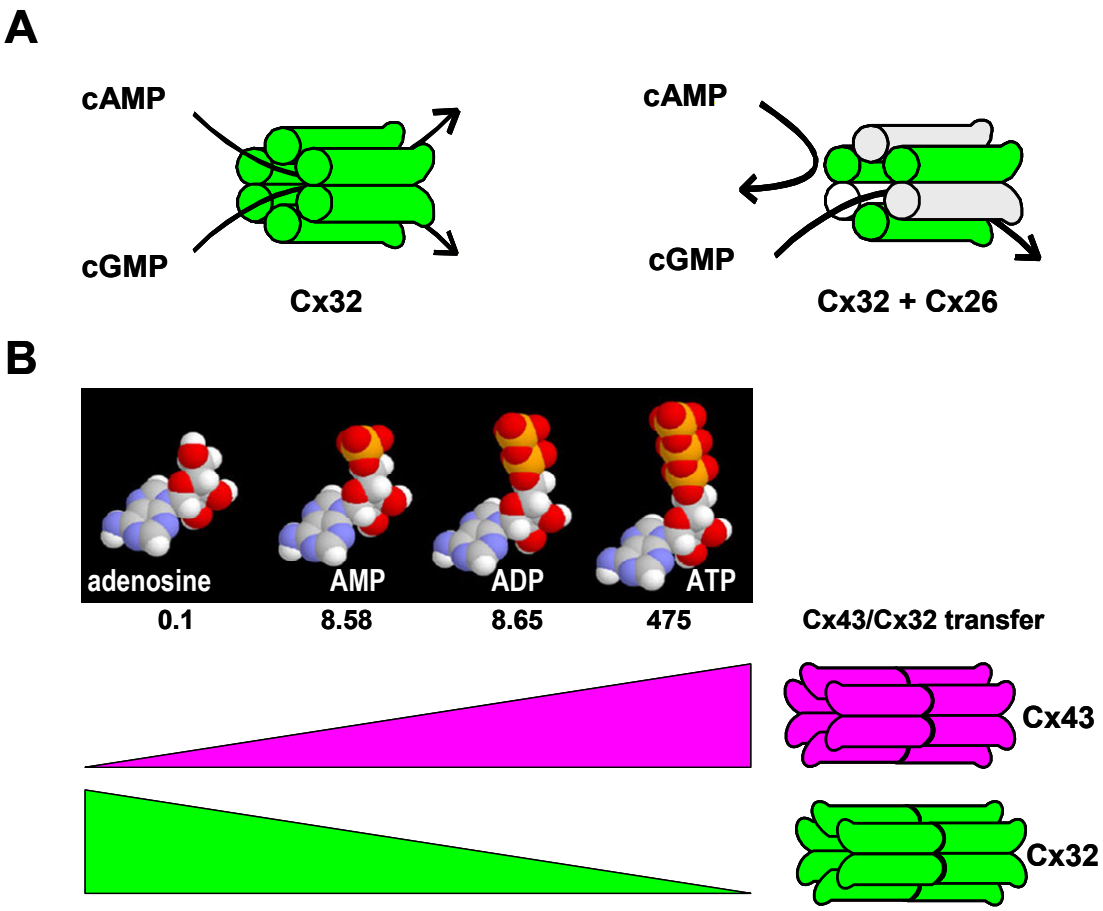

A
cAMP
cGMP
Cx32
cAMP
cGMP
Cx32 + Cx26
B
adenosine
AMP
ADP
ATP
0.1
8.58
8.65
475
Cx43/Cx32 transfer
Cx43
Cx32

Figure I-3. Consequences of pathogenic connexin mutations on gap junction biogenesis and function. Pathogenic mutations not only affect gap junction channel formation at different levels from protein translation to assembly of gap junctions but can also alter the channel function. Skin disease associated connexin mutations have been shown to execute their action through three main mechanisms. They may inhibit protein trafficking to the plasma membrane, therefore impairing connexon/gap junction channel assembly (1). They may exert a dominant-negative action on co-expressed connexins, changing the types of molecules exchanged between the cells (2). Finally, they may result in the production of constitutively active hemichannels (3), leading to the unregulated release of cellular contents into the extracellular space (Mese et al., 2007).

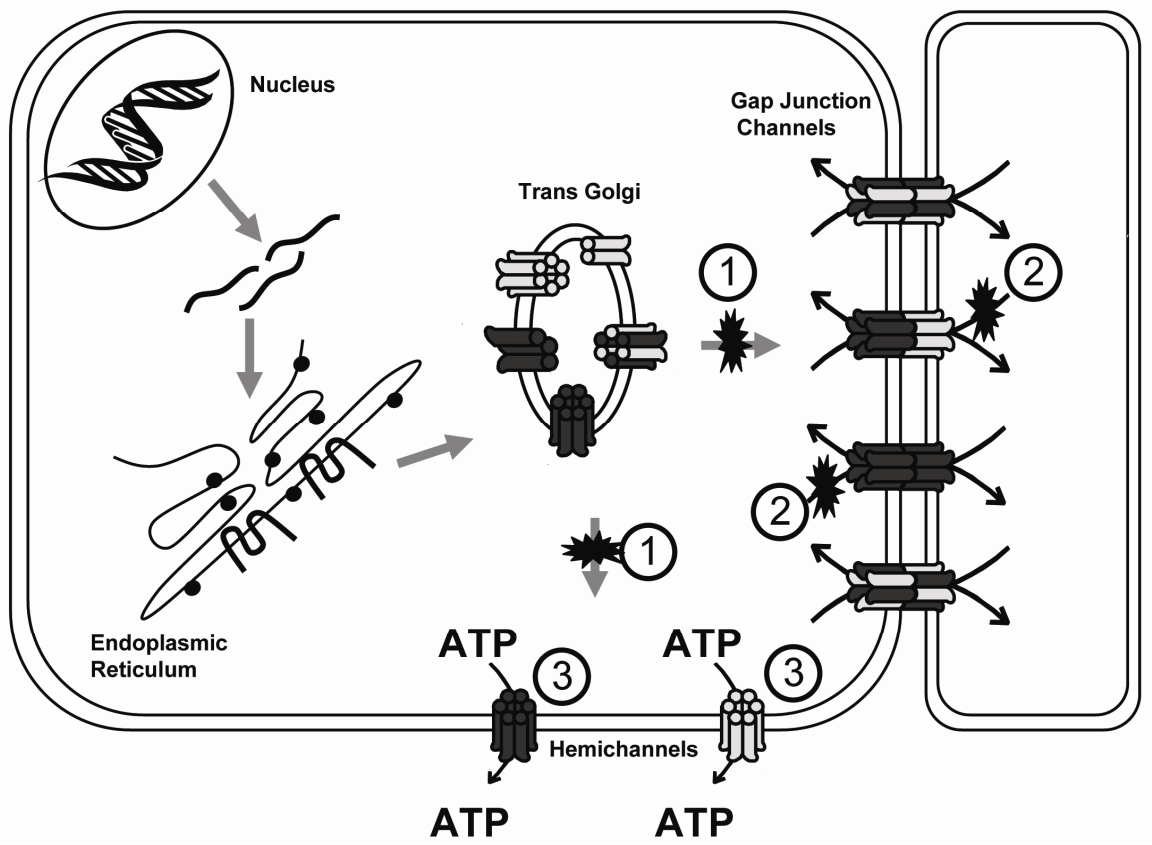

Nucleus
Gap Junction
Channels
Trans Golgi
1
2
2
1
Endoplasmic
Reticulum
ATP
3
ATP
3
Hemichannels
ATP
ATP

Figure I-4. Anatomy of the cochlea. There are two fluid filled environmentS in the cochlea, endolymph and perilymph which have different ionic compositions. Endolymph has high potassium concentration and a positive 80-100 mV potential difference relative to the perilymph. On the other hand, perilymph has high sodium and low potassium. The organ of Corti is the hearing organ and consists of two main cell types, the sensory hair cells and the non-sensory supporting cells. The movement of the tectorial membrane activates potassium channels on the hair cells allowing K^+ influx from the endolymph. Hair cells then releases these ions into the extracellular space from which the surrounding supporting cells take up K^+ and recycles it back to the endolymph by means of cochlear gap junction networks. Therefore, the sensitivity of hair cells and the high potassium concentration in the endolymph are maintained (Teubner et al., 2003).

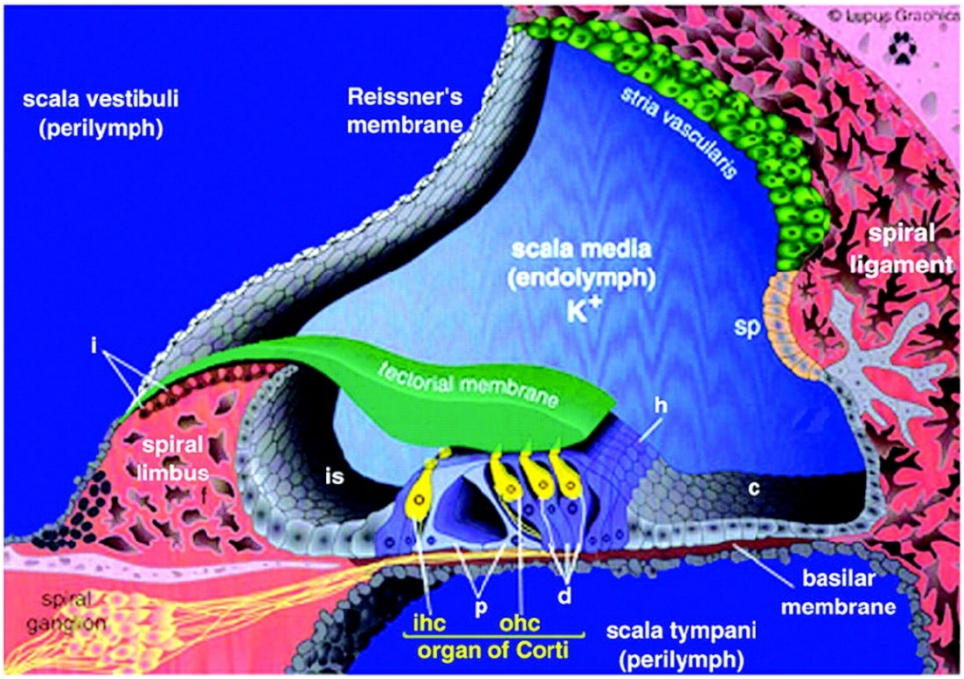

© Lupus Graphica
scala vestibuli
(perilymph)
Reissner's
membrane
stria vascularis
scala media
(endolymph)
K+
spiral
ligament
sp
i
tectorial membrane
h
spiral
limbus
is
c
basilar
membrane
spiral
ganglion
p
d
ihc
ohc
organ of Corti
scala tympani
(perilymph)

Figure I-5. The role of gap junction networks in the inner ear: Potassium ion re-circulation. Gap junctional communication is important for the removal of potassium ions around hair cells and their re-circulation back to the endolymph (Kikuchi et al., 2000a;Zhao et al., 2006). After the initiation of hearing process, the surrounding supporting cells (SC) take up K^+ around hair cells (HC) and carry them to the lower level of the spiral ligament through the epithelial cell gap junction network (RC, root cells). Here, type II fibrocytes (FC) pick these ions and transfer them to the cells in the stria vascularis by means of connective tissue cell gap junction network. The marginal cells (MC) then pumps K^+ back to the endolymph. In the case of connexin associated mutations, the potassium circulation pathway would be impaired, resulting in excess K^+ around hair cells. This might cause cell death and play a role in the generation of hearing loss. BC, basal cells and IC, intermediate cells.

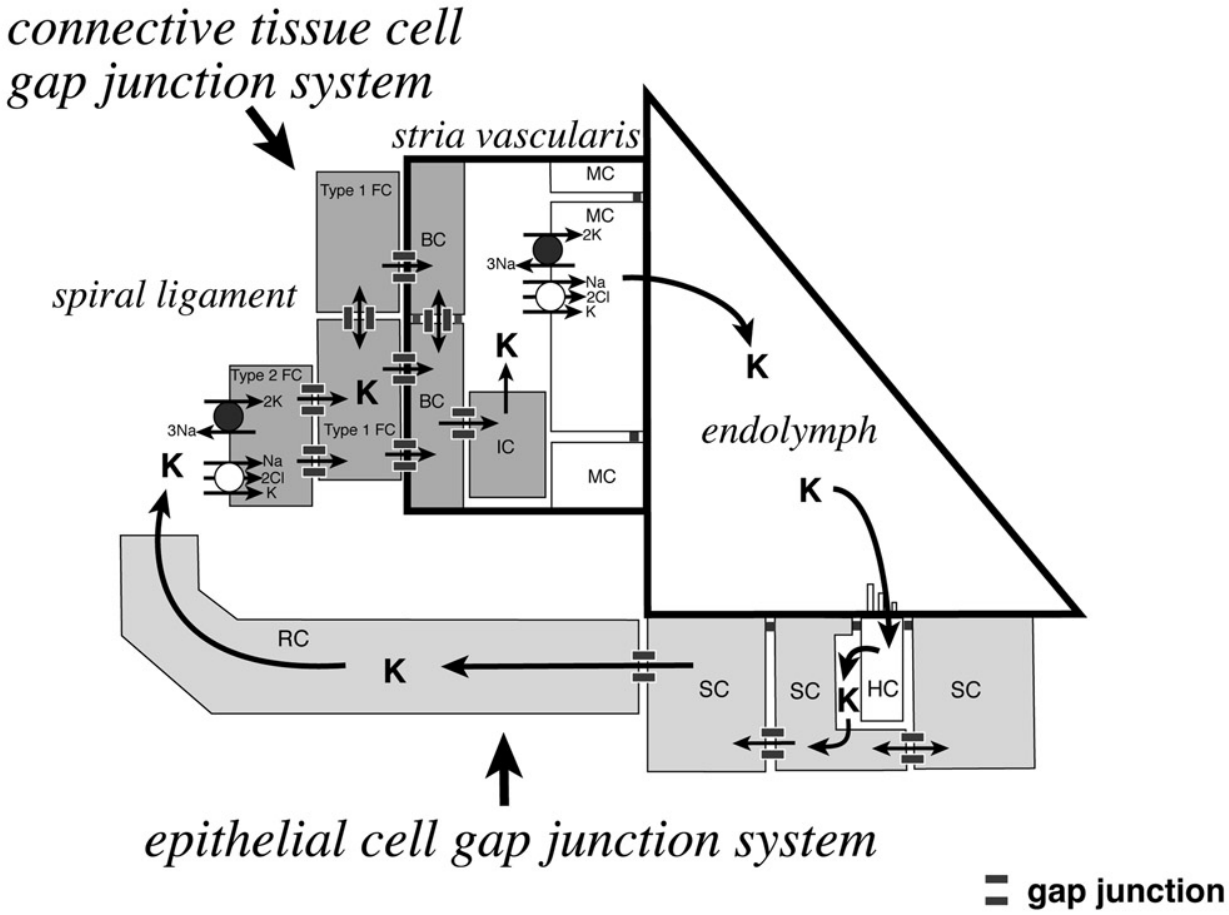
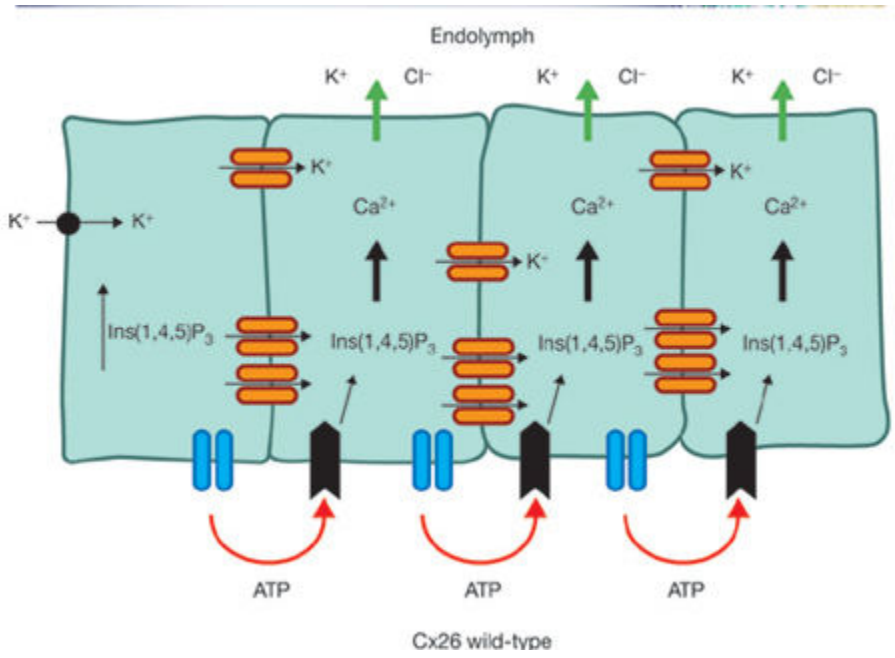

connective tissue cell
gap junction system
stria vascularis
spiral ligament
Type 1 FC
BC
MC
MC
2K
3Na
Na
2Cl
K
K
Type 2 FC
2K
3Na
K
Na
2Cl
K
BC
Type 1 FC
IC
MC
K
endolymph
K
RC
K
SC
SC
K
HC
SC
epithelial cell gap junction system
gap junction

Figure I-6. The role of biochemical coupling through cochlear gap junction channels during hearing processes. In addition to K^+, the second messenger IP_3 can also pass through gap junction channels. The transfer of IP_3 is one of the mechanisms that can generate Ca^{2+} waves in the supporting cells. This mechanism was speculated to remove excess K^+ by the activation of K^+/Cl^- efflux system (Bruzzone and Cohen-Salmon, 2005).



Cx26 wild-type

Table I-1. Cochlear and epidermal connexins and associated disorders. The association of connexins with different human hereditary diseases has implicated their importance for tissue homeostasis. This table summarizes the expression pattern of connexins in both the cochlea and skin and their associated diseases.

GENE	HEREDITARY DISEASE	OMIM REFERENCE	EXPRESSION PATTERN
GJB2(Cx26)	Autosomal recessive non-syndromic sensorineural hearing loss (DFNB1)	220290	Almost ubiquitous, including Cochlea, Skin, Liver, Placenta, Breast, Lung, Brain
	Autosomal dominant non-syndromic sensorineural hearing loss (DFNA3)	601544	
	Vohwinkel syndrome	124500	
	Keratitis-Ichthyosis-Deafness (KID) syndrome	148210	
	Palmoplantar keratoderma (PPK) associated with sensorineural hearing loss	148350	
	Bart-Pumphrey syndrome	149200	
GJB3(Cx31)	Autosomal dominant and recessive Erythrokeratodermia variabilis (EKV)	133200	Skin, Cochlea, Placenta, Kidney, testes, Eye, PNS
	Autosomal dominant and recessive non-syndromic sensorineural hearing loss (DFNA3)	600101	
GJB4(Cx30.3)	Autosomal dominant Erythrokeratodermia variabilis (EKV)	133200	Skin, kidney, placenta
GJB6(Cx30)	Autosomal recessive non-syndromic sensorineural hearing loss (DFNB1)	220290	Skin, Brain, Cochlea, Cornea
	Autosomal dominant non-syndromic sensorineural hearing loss (DFNA3)	601544	
	Clouston Syndrome (Hidrotic ectodermal dysplasia, HED)	129500	
GJA1(Cx43)	Oculo-dento-digital dysplasia (ODDD)	164200	Ubiquitous, including Skin, Heart, Eye, Brain

Chapter II: Altered Gating Properties of Functional Cx26 Mutants Associated with Recessive Non-syndromic Hearing Loss

Abstract:

Connexins (Cx) form gap junctions that allow the exchange of small metabolites and ions. In the inner ear, Cx26 is the major gap junction protein and mutations in the Cx26-encoding gene, *GJB2*, are the most frequent cause of autosomal recessive non-syndromic hearing loss (DFNB1). We have functionally analyzed five Cx26 mutations associated with DFNB1, comprising the following single amino-acid substitutions: T8M, R143W, V153I, N206S and L214P. Coupling of cells expressing wild-type or mutant Cx26 was measured in the paired *Xenopus* oocyte assay. We found that the R143W, V153I and L214P mutations were unable to form functional channels. In contrast, the T8M and N206S mutants did electrically couple cells, though their voltage gating properties were different from wild-type Cx26 channels. The electrical coupling of oocytes expressing the T8M and N206S mutants suggest that these channels may retain high permeability to potassium ions. Therefore, deafness associated with Cx26 mutations may not only depend on reduced potassium re-circulation in the inner ear. Instead, abnormalities in the exchange of other metabolites through the cochlear gap junction network may also produce deafness.

Introduction

Gap junctional communication has been implicated in several hereditary human diseases, like cataracts, X-linked Charcot-Marie Tooth disease, skin disorders and sensorineural hearing loss (Kelsell et al., 1997;Rabionet et al., 2002;White and Paul, 1999). Linkage of connexin gene mutations to these diverse pathologies improves understanding of the in vivo functions of connexins. Expression patterns of several connexins have been described in the inner ear, where they play important roles in several processes of auditory transduction, such as recycling of K^+ ions in the endolymph and exchange of metabolites between supporting cells (Kelley et al., 2000;Kikuchi et al., 1995;Forge et al., 2003;Bruzzone et al., 2003;Richard et al., 1998b). Cx26, Cx30 and Cx31 are all expressed in the cochlear supporting cells, and mutations in these connexins have been associated with both syndromic and non-syndromic forms of genetic deafness (Marziano et al., 2003;Petit et al., 2001;Rabionet et al., 2000;Bruzzone et al., 2003) (http://davinci.crg.es/deafness/).

Sensorineural deafness affects one child in 1,000 at birth and 35–40% of these cases have a genetic basis (Kenna et al., 2001;Lefebvre and Van De Water, 2000;Rabionet et al., 2000). Approximately 80% of genetic deafness is of the non-syndromic form, presenting with impaired hearing and no other clinical features (Estivill et al., 1998;Kenna et al., 2001;Kenneson et al., 2002). Mutations in the *GJB*2 locus were found to be associated with pathogenesis of most autosomal recessive non-syndromic hearing loss (DFNB1), autosomal dominant non-syndromic hearing loss (DFNA3) and sporadic congenital deafness (Kenneson et al., 2002;Estivill et al., 1998;Kelsell et al.,

1997;Rabionet et al., 2000). The mutations involved in deafness include nonsense or insertion/deletion, which results in the premature termination of protein translation, and missense mutations that alter a single amino acid. The most frequently found mutation in the Cx26 gene, *GJB*2, involved in 70–85% of Cx26-related deafness is 35delG (Zelante et al., 1997). Deletion of this one base results in a frameshift, introducing a premature stop codon and resulting in termination of protein translation. In addition to 35delG, over 50 missense mutations have been identified throughout *GJB*2 (http://davinci.crg.es/deafness/) and some of them have been analyzed for functional properties. For example, Bruzzone et al. have analyzed the F83L polymorphism and V84L mutation, and they have shown that this mutant and polymorphism were able to form functional channels, and that voltage-gating properties of F83L, V84L and wild-type Cx26 channels shared similar features (Bruzzone et al., 2003). In another study by D'Andrea et al. , six more Cx26 mutations have been analyzed, and G12V, S19T, M34T and R184P were not able to form active channels in transfected HeLa cells, while L90P and R127H showed very low levels of Lucifer Yellow transfer (D'Andrea et al., 2002). Thus, not all deafness-causing mutations resulted in loss of activity and the functional mutations may provide insights into the specific function of the Cx26 gene in hearing.

Five Cx26 missense mutations [T8M, R143W, V153I, N206S and L214P (Brobby et al., 1998;Hamelmann et al., 2001;Kenna et al., 2001;Marlin et al., 2001)] associated with nonsyndromic hearing loss were analyzed in order to identify mutants that remain functional. Their ability to form functional channels was tested in the paired Xenopus oocyte expression system. Three of these mutants were unable to form functional

channels, while the other two formed partially functional channels with altered voltage-gating properties.

Materials and methods

Molecular cloning

Wild-type human Cx26 (Accession; NM_004004) was subcloned into the XbaI-BamHI sites of the pCS2+ expression vector for functional expression studies in Xenopus oocytes. The following mutations were introduced using standard oligonucletide directed mutagenesis: L214P, N206S, R143W, T8M and V153I, and then subcloned into the pCS2+ vector. Mutated constructs were sequenced on both strands.

In vitro transcription, oocyte microinjection and pairing

All constructs were linearized using NotI, and the Message Machine kit (Ambion) was used to transcribe RNAs. Adult *Xenopus* females were anesthetized and ovarian lobes were surgically removed. To collect stage V–VI oocytes, lobes were defollicullated in a solution containing 50 mg/ml collagenase B and 50 mg/ml hyaluronidase in modified Barth's medium (MB) without Ca^{2+}. Cells were subsequently cultured in MB medium at room temperature. For physiological analysis, cells were first injected with 10 ng of an antisense Xenopus Cx38 oligonucleotide to eliminate the conductance of endogenous intercellular channels. After a 24-h incubation, antisense oligonucleotide-injected oocytes were re-injected with specified Cx26 RNAs (5 ng/cell) or H_2O as a negative control. Subsequently, vitelline envelopes were removed in a hypertonic solution, and the oocytes

were manually paired with the vegetal poles apposed in MB medium containing Petri dishes.

Dual-cell voltage clamp

Following an overnight incubation, gap junction coupling between oocyte pairs was measured using dual-cell voltage-clamp techniques (Spray et al., 1991;Bruzzone et al., 2003). Current and voltage electrodes (1.2 mm diameter, omega dot; Glass Company of America, Millville, N.J., USA) were pulled to a resistance of 1–2 MΩ with a horizontal puller (Narishige, Tokyo, Japan) and filled with a solution containing 3 M KCl, 10 mM EGTA and 10 mM HEPES, pH 7.4. Voltage clamping of oocyte pairs was performed using two GeneClamp 500 amplifiers (Axon Instruments, Foster City, Calif., USA) controlled by a PC-compatible computer through a Digidata 1320A interface (Axon Instruments). The pCLAMP 8.0 software (Axon Instruments) was used to program stimulus and data collection paradigms. Current outputs were filtered at 50 Hz and the sampling interval was 10 ms. For simple measurements of junctional conductance, both cells of a pair were initially clamped at –40 mV to ensure zero transjunctional potential and alternating pulses of ±20 mV were imposed to one cell. Current delivered to the cell clamped at –40 mV during the voltage pulse was equal in magnitude to the junctional current, and was divided by the voltage to yield the conductance.

To determine voltage-gating properties, transjunctional potentials (V_j) of opposite polarity were generated by hyperpolarizing or depolarizing one cell in 20 mV steps (over a range of ±120 mV), while clamping the second cell at –40 mV. Currents were measured

at the end of the voltage pulse, at which time they approached steady state (I_{jss}) for the majority of tested voltages, and the macroscopic conductance (G_{jss}) was calculated by dividing I_{jss} by V_j. G_{jss} was then normalized to the values determined at ±20 mV, and plotted against V_j. Data describing the relationship of G_{jss} as a function of V_j were analyzed using Origin 6.0 (Microcal Software, Northampton, Mass., USA) and fit to a Boltzmann relation of the form: $G_{jss}=(G_{jmax}–G_{jmin})/1+\exp[A(V_j–V_0)]+G_{jmin}$, where G_{jss} is the steady-state junctional conductance, G_{jmax} (normalized to unity) is the maximum conductance, G_{jmin} is the residual conductance at large values of V_j, and V_0 is the transjunctional voltage at which $G_{jss}=(G_{jmax}–G_{jmin})/2$. The constant A (=nq/kT) represents the voltage sensitivity in terms of gating charge as the equivalent number (n) of electron charges (q) moving through the membrane, k is the Boltzmann constant, and T is the absolute temperature.

Preparation of oocyte samples for Western blot analysis

Oocytes were collected and lysed in 1 ml of lysis buffer containing 5 mM Tris pH 8.0, 5 mM EDTA and protease inhibitors (White et al. 1992). The oocytes were sheared using a 1-ml syringe and a series of needles of diminishing caliber (20, 22, 26 Ga). After homogenization extracts were centrifuged at 1,000 g at 4°C for 5 min in a microcentrifuge. The supernatant was transferred to a new tube and centrifuged at 100,000 g (45,000 rpm) at 4°C for 30 min in a TLC45 ultracentrifuge. Membrane pellets were dissolved in SDS sample buffer and samples were separated on 15% SDS gels and then transferred to nitrocellulose membranes. Blots were blocked with 3% BSA in PBS

with 0.02% NaN_3 for 1 h and then probed with polyclonal anti-rat Cx26 antibody (Zymed) at a 1:1,000 dilution. Band intensities were quantified using Kodak 1D Image Analysis software (Eastman Kodak, Rochester, N.Y., USA). Values from three independent experiments were normalized to the mean value of band intensity of the wild-type Cx26 sample. Statistical analyses between wild-type Cx26, T8M and N206S levels were performed using the one-way ANOVA test with a significance level of 0.05.

Results

Formation of functional gap junction channels by T8M and N206S

We have used the Xenopus oocyte expression system to analyze the ability of Cx26 mutations associated with DFNB1 to form functional gap junction channels. The missense mutations, T8M, R143W, V153I, N206S, and L214P as depicted in Fig. II-1 were tested. Oocytes were pretreated with an antisense oligonucleotide against Xenopus Cx38 to reduce the background conductance provided by endogenous gap junctions in this system (Barrio et al., 1991;Bruzzone et al., 1993). After mutant or wild-type Cx26 cRNA injection, oocytes were paired and intercellular currents were quantified and analyzed. Water injected antisense oligonucleotide treated cells were used as negative controls.

As expected, wild-type Cx26 formed functional channels with junctional conductance that was 100-fold higher than background levels recorded in antisense-oligonucleotide-injected control pairs (Fig. II-2A). In contrast, the junctional conductances of the R143W, V153I and L214P mutants were equal to or less than background levels ($P>0.05$, Student's unpaired t-test), establishing the inabilities of these mutants to form functional intercellular channels (Fig. II-2A). Oocyte pairs injected with the two remaining mutants, T8M and N206S, developed junctional coupling above background levels, N206S had a mean conductance five-fold higher than background, while T8M conductance was twofold higher than negative controls ($P<0.05$, compared with antisense oligonucleotide injected controls). However, both functional mutants had

macroscopic conductance significantly lower than wild-type Cx26 ($P<0.05$, compared with Cx26). A scatter plot of all the data revealed that the incidence of coupling above background was 57% for N206S, and 31% for T8M, both of which were lower than the 100% coupling incidence of wild-type Cx26 (Fig. II-2B). Thus the T8M and N206S mutations retained the ability to form functional gap junction channels, but with a reduction in both the magnitude and incidence of coupling compared with wild-type Cx26.

Synthesis of mutant connexins in Xenopus oocytes

The reduction of intercellular coupling for T8M and N206S may have resulted from reduced levels of protein synthesis or stability for these mutated Cx26 isoforms. To analyze translation of the injected RNAs, oocytes that had received equal quantities of wild-type Cx26, T8M and N206S cRNAs were collected after dual-cell voltage clamp and analyzed by Western blot using a Cx26-specific antibody. Immunoblot analysis of Xenopus oocytes injected with the specified RNAs showed that both mutated constructs were expressed in oocytes, at similar levels to wild-type Cx26 (Fig. II-3A). This observation was verified by plotting the mean densitometry values of replicate blots ($n=3$) which failed to reveal any significant differences (one-way ANOVA, $P<0.05$) between the expression levels of wild-type Cx26, or the T8M and N206S mutations (Fig. II-3B). These results suggest that the reductions in the incidence and magnitude of coupling were not simply due to reduced protein translation or stability with the two functional mutant constructs.

Voltage-gating properties of T8M and N206S channels

We next analyzed the gating properties of the two functional mutants, N206S and T8M. To achieve this goal, we examined the response of wild-type, N206S and T8M to a range of hyperpolarizing and depolarizing transjunctional voltages. Figure II-4 shows the junctional currents (I_j) and the relationship between steady-state junctional conductance (G_j) and transjunctional voltage (V_j) for wild-type Cx26 and the two mutants. As previously reported (Barrio et al., 1991;Bruzzone et al., 2003), at potentials greater than ±80 mV, junctional currents of wild-type Cx26 pairs decayed slowly and asymmetrically over the course of the voltage step (Fig. II-4A). In contrast, the T8M and N206S channels illustrated altered gating characteristics from wild-type Cx26. For N206S, the decay in I_j was similar to Cx26, but appeared qualitatively slower than wild-type (Fig. II-4C). Similar to wild-type Cx26, N206S pairs also showed asymmetry in their gating. The I_j of T8M pairs had very distinctive characteristics. For positive V_j values, the junctional currents initially increased over the duration of the voltage step, showing a slight decay only at the highest values of V_j. For negative values of V_j, the junctional current also increased over the time of the voltage step (Fig. II-4E).

To quantify differences in the magnitude of the voltage gating, conductance values at the end of the imposed pulse were normalized, plotted against V_j and were fit to a Boltzmann equation (Table II-1). These data revealed that for wild-type Cx26 the transjunctional voltage required to yield a conductance midway (V_0) between maximum (G_{jmax}) and minimum conductance (G_{jmin}) had a value of 98 mV for positive V_js, and a

value of –95 mV for negative V_js. Conversely, the V_0s for N206S were slightly larger, 116 mV for positive values and –123 mV for negative values, consistent with a reduced sensitivity to voltage for this mutant channel. As the T8M data were not possible to fit with the Boltzmann equation, we could not quantify its voltage dependence.

To quantify differences in the kinetics of the voltage gating, I_js were plotted against time and fit to a single exponential decay equation. The mean time constant (τ) for the rate of decline in I_j over time for wild-type Cx26 at a V_j of +120 mV was 0.99±0.07 s (mean±SE, n=11, Fig. II-5A). In contrast, N206S channels closed significantly slower at V_j of +120 mV, the average time constant for channel closure was 1.99±0.26 s (n=7, $P<0.05$, Fig. II-5B). As the T8M currents were not possible to fit with the exponential decay equation, we could not quantify the kinetics of its voltage dependent closure. In any case, these data clearly show that the magnitude and kinetics of the voltage-gating properties of T8M and N206S both differ quantitatively from those of wild-type Cx26 channels.

Discussion

Functional analysis of Cx26 mutations linked to deafness may improve the understanding of the role played by intercellular communication in cochlear homeostasis. Our hypothesis is that deafness caused by mutations in Cx26 is not due to a simple loss of ionic junctional communication, but rather by changes in junctional permeability between cochlear cells induced by selective loss of one out of the three connexin isoforms normally available. Thus, one can predict that a subset of the missense Cx26 mutations causing deafness may retain ionic coupling, but show altered permeability to larger solutes. Indeed, such mutations could provide valuable tools for probing the type of molecules cochlear cells need to share to preserve organ homeostasis and prevent hearing loss. Toward this goal, we have analyzed five Cx26 missense mutations associated with DFNB1 to identify disease-causing mutants that retained functional activity. We found that while the R143W, V153I and L214P mutants completely lost their abilities to form functional gap junction channels, the T8M and N206S mutants retained the ability to provide intercellular communication, although at lower conductance levels, and with gating properties that differed from wild-type Cx26 channels.

Many deafness associated missense Cx26 mutations studied thus far resulted in a complete loss of ability to form functional channels (Martin et al., 1999;White et al., 1998). These studies indicated that the failure of many mutants to form functional channels depended on improper targeting to the plasma membrane, reduced protein stability, failure to oligomerize into hemichannels, or impaired docking into complete channels(Thomas et al., 2004;Thonnissen et al., 2002;White, 2000). Three of the five

mutants we tested failed to induce intercellular coupling in paired Xenopus oocytes. Our biochemical analysis showed that all of the mutants were expressed at similar levels to wild-type Cx26 (Fig. II-3, and data not shown). Thus the inability to form functional channels was not likely a result of reduced protein expression. Further experimentation will be required to determine at which level of assembly these mutations fail; possibilities include altered trafficking, reduced unitary conductance, or reduced open probability of the channels. Further investigation of these same parameters may also help to explain why the T8M and N206S mutations exhibited a reduced magnitude of coupling despite showing similar levels of synthesis to wild-type Cx26.

Our data demonstrated that the V153I mutation was unable to form functional channels. However, this mutation has been alternatively reported as either a polymorphism or a recessive pathogenic allele. Marlin et al. identified the V153I mutation in normal-hearing parents in association with 35delG mutation on the other allele, so that they considered the V153I change as a polymorphism (Marlin et al., 2001). In addition, Bayazit et al. claimed V153I as a polymorphism rather than a pathogenic change (Bayazit et al., 2003). In contrast, Wu et al. identified this mutation in deaf subjects in a compound heterozygous state with the T8M missense mutation and proposed it to be a pathogenic mutation (Wu et al., 2002). Moreover, Rickard et al. identified a deaf woman homozygous for V153I, although linkage analysis has not yet established pathogenicity (Rickard et al., 2001). Our study may support the view that V153I can be a pathogenic change, as V153I mutants could not form functional channels, producing junctional conductance lower than background levels. R143W and L214P have been shown to be pathogenic, as two studies have identified deaf subjects carrying

homozygous R143W missense mutations and the R143W mutation as compound heretozygous with L214P (Brobby et al., 1998;Hamelmann et al., 2001). This is consistent with the results in this study as these mutants had junctional conductance less than or equal to background levels.

A few Cx26 mutations have previously been reported to retain some level of functional properties (D'Andrea et al., 2002;Wang et al., 2003;Bruzzone et al., 2003). Here, the T8M and N206S mutations displayed some level of functional activity, having junctional conductance higher than background levels. Several studies indicated that these mutants are pathogenic (Kenna et al., 2001;Marlin et al., 2001;Wu et al., 2002) and are inherited in an autosomal recessive manner (Kenna et al., 2001). As described above, T8M was found in compound heterozygotes with the V153I missense mutation in deaf subjects (Kenna et al., 2001;Marlin et al., 2001). Patients carrying the N206S mutation were either heterozygous for this change or in compound heterozygotes state with 35delG or V37I (Kenna et al., 2001;Marlin et al., 2001;Wu et al., 2002). In these studies, patients carrying the N206S mutation were observed to exhibit mild hearing loss, causing the authors to speculate that this mutation may not severely compromise the gap junctional communication system in the inner ear (Kenna et al., 2001). This is consistent with our results, as the N206S mutants show similar voltage-gating properties to wild-type channels.

In the inner ear, there are two main gap junctional systems: the non-sensory epithelial cell system including cochlear supporting cells and root cells, and the connective tissue cell system in the cochlear lateral wall comprised of fibrocytes in the spiral ligament, and cells in the stria vascularis (Forge et al., 2002;Kikuchi et al., 2000a).

These gap junction systems form a network that functionally couples these cells in the inner ear and is thought to provide the re-circulation of K^+ ions back to the endolymph after activation of auditory transduction (Forge et al., 2002;Kikuchi et al., 2000a;Kemperman et al., 2002;Wilcox et al., 2000). Cx26 expression corresponded to the distribution of gap junctions throughout the inner ear (Kikuchi et al., 1994;Kikuchi et al., 2000b). In addition, Cx30 and Cx31 were also expressed in the inner ear, and Cx30 co-localizes with Cx26 (Marziano et al., 2003;Rabionet et al., 2000). Mutations in these connexin genes were also found to be involved in genetic deafness (Marziano et al., 2003;Rabionet et al., 2000;Petit et al., 2001;Bruzzone et al., 2003).

Our results indicated that some of the Cx26 mutants were still able to form functional channels since the junctional conductance of T8M and N206S was higher than that of water-injected oocytes. Given that T8M and N206S channels were electrically coupled, and that potassium is the dominant permeant ion of cytoplasm, it is likely that these mutant channels retain high permeability to K^+ ions, as has been demonstrated for all gap junction channels (Valiunas et al., 2002). Thus, deafness associated with these missense mutations in the Cx26-encoding gene may not solely depend on the loss of K^+ coupling and K^+ re-circulation. Instead, these mutations may interfere with the coupling of larger biochemical solutes in the inner ear. Cohen-Salmon et al. have shown that targeted deletion of Cx26 in the inner ear resulted in degeneration of cells within the cochlea, including inner and outer hair cells and in distortion of the organ of Corti (Cohen-Salmon et al., 2002). However, Cx30 distribution and expression within the inner ear was normal in these mice. Furthermore, Cx26 and Cx30 have similar functional properties and Kudo et al. suggested that connexins especially Cx30 in the cochlea might

compensate the K^+ cycling since K^+ can also efficiently pass through these junctional channels (Valiunas et al., 1999a;Kudo et al., 2003). Therefore, hearing loss resulting from functional Cx26 mutations may be due to failure in the passage of other small metabolites or secondary messengers rather than K^+.

Many studies have shown that gap junctional channels formed by different connexins have distinctive permeability for larger solutes like cyclic nucleotides and inositol phosphates (Manthey et al., 2001;Valiunas et al., 2002). In addition, Manthey et al. documented permeation differences between Cx26 and Cx30, showing that they display differences in the diffusion of dyes of the same size and charge as the larger metabolites (Manthey et al., 2001). Therefore, loss of Cx26 in the cochlea may alter the exchange of certain metabolites or secondary messengers between cells in the inner ear. Further study of partially functional channels formed by N206S and T8M mutants may improve the characterization of molecules exchanged within the gap junctional systems in the inner ear that fail to pass through the mutant gap junctions resulting in deafness. One potential candidate molecule is implied by recent studies of mice with a conditional inactivation of Cx26 in the inner ear, suggesting that Cx26 may play an essential role in preventing the accumulation of glutamate that leads to apoptotic cell death (Cohen-Salmon et al., 2002). Glutamate is normally taken up by the supporting sensory epithelium and the inner hair cells, and could be spatially buffered through the Cx26 gap junctional syncitium as has been proposed for K^+ ions. Demonstration of a reduction in glutamate permeability in the functional Cx26 mutants awaits further experimentation.

Figure Legends and Figures:

Figure II-1. Schematic representation of Cx26 relative to the plasma membrane showing the missense mutations analyzed in this dissertation. The arrows indicate the five missense mutations analyzed, T8M, R143W, V153I, N206S and L214P. T8M is in the N-terminus, R143W and V153I are located on the third transmembrane domain and N206S and L214P are on the fourth transmembrane domain.

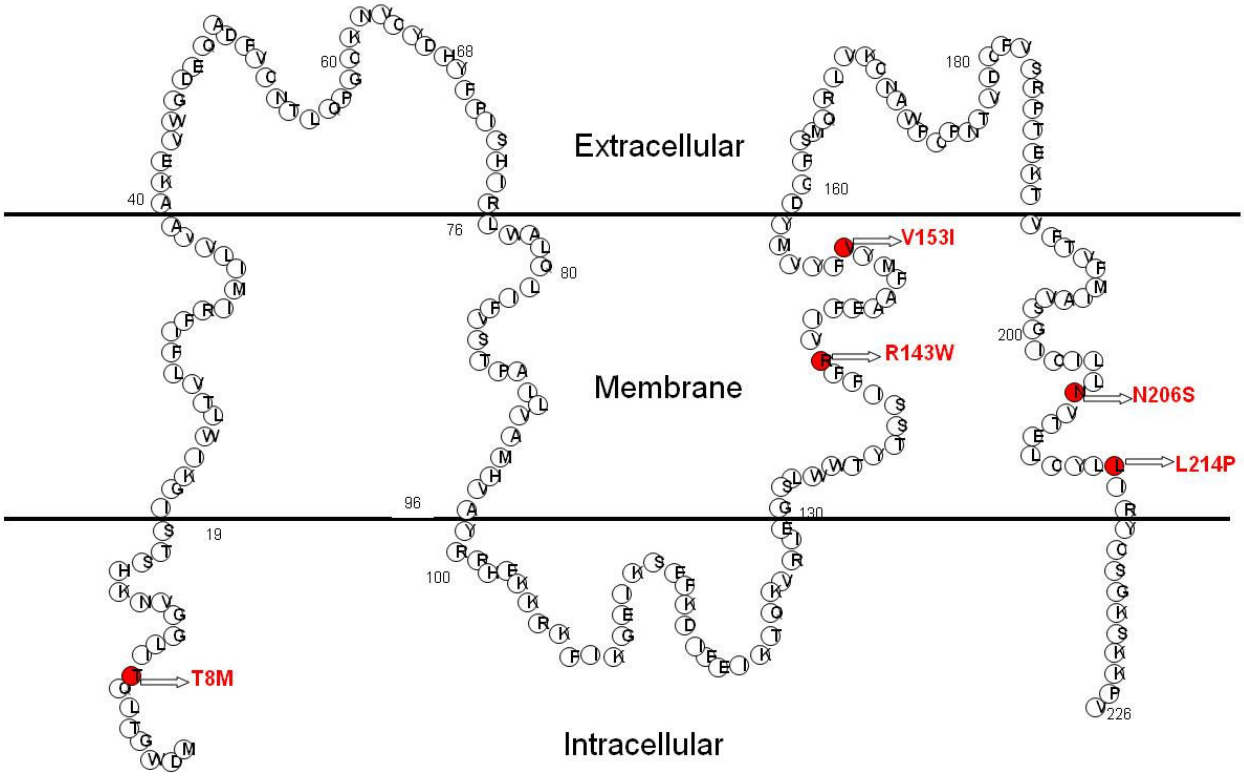

Extracellular
Membrane
Intracellular
T8M
V153I
R143W
N206S
L214P

Figure II-2. Analysis of the ability of Cx26 mutants associated with DFNB1 to form functional gap junction channels. A. The junctional conductance of wild-type Cx26 (n=43), T8M (n=38), R143W (n=10), V153I (n=15), N206S (n=30) and L214P (n=12) mutants were compared with that of antisense oligonucleotide injected control oocytes (n=43). R143W-, V153I- and L214P-injected cell pairs produced junctional conductance equal to or less than the background levels measured in antisense oligonucleotide treated control pairs. Wild-type Cx26 produced junctional conductance 100-fold higher than background, while T8M and N206S showed lower junctional conductance levels than wild-type channels, but still twofold and five-fold higher than background level, respectively. **B.** Scatter plot showing the incidence of coupling for wild-type Cx26, T8M and N206S channels. The dashed line indicates the mean level of background conductance. For wild type, the incidence of coupling was 100% and the magnitude of coupling was higher than either mutant. In contrast, the incidence of coupling higher than background was 31% for T8M and 57% for N206S.

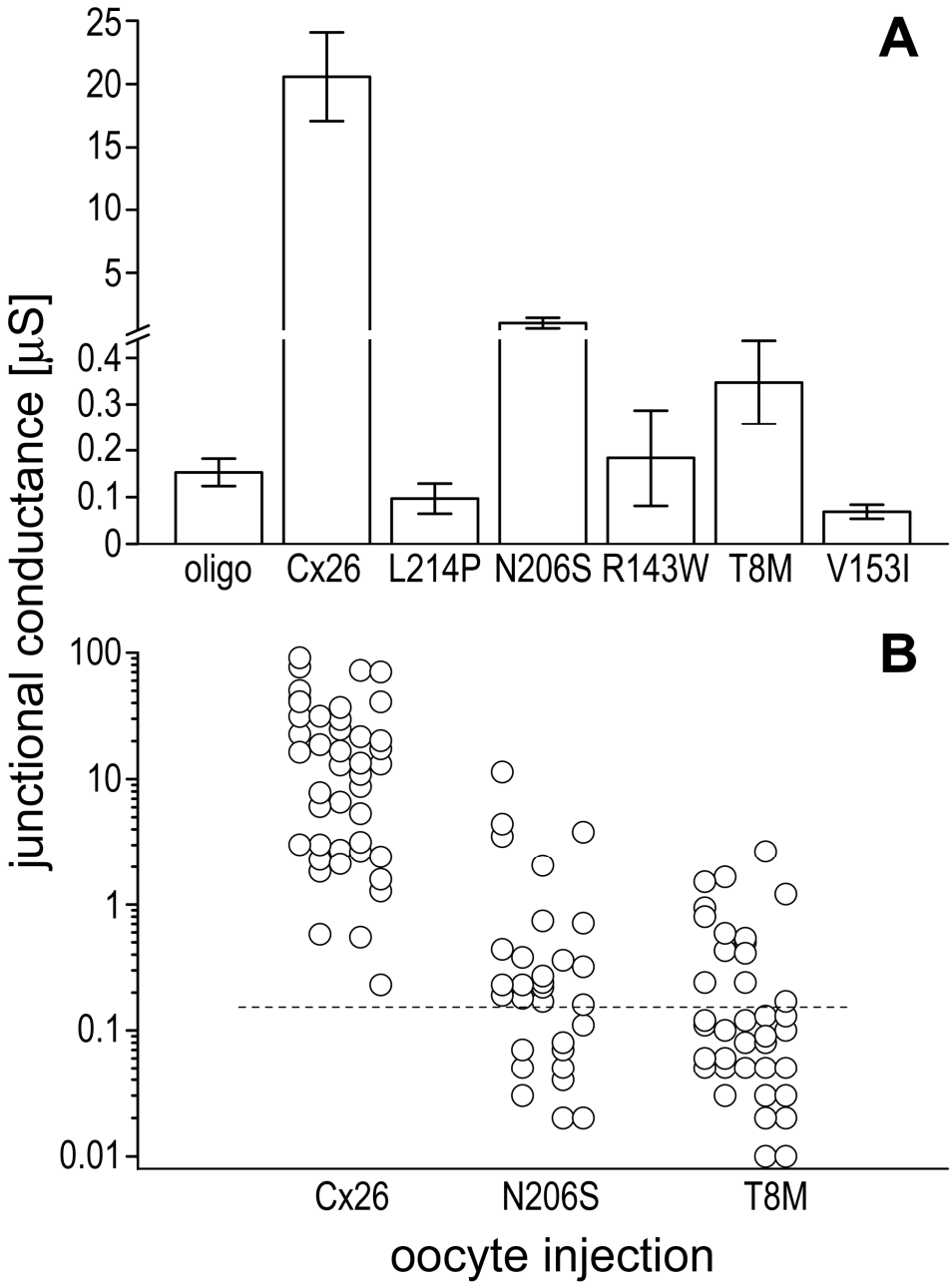

A
25
20
15
10
5
0.4
0.3
0.2
0.1
0
junctional conductance [µS]
oligo
Cx26
L214P
N206S
R143W
T8M
V153I
B
100
10
1
0.1
0.01
Cx26
N206S
T8M
oocyte injection

Figure II-3. Immunoblot analysis of Xenopus oocyte extracts indicates that wild-type and mutant connexins are equally synthesized. A. Equal numbers of oocytes from each cRNA-injected group were pooled and membrane fractions were prepared. Following resuspension in sample buffer, 20% of the preparation was loaded in each lane for each condition. The Western blot was probed with an affinity-purified rabbit anti-Cx26 antibody. Despite resulting in both a lower magnitude and reduced incidence of coupling, the T8M and N206S mutants had similar expression levels to wild-type Cx26 protein. **B.** Quantitation of wild-type Cx26, N206S and T8M expression. Relative synthesis of wild-type and mutant connexins were not statically different ($P>0.05$). Values are the mean±S.E. of three independent experiments.

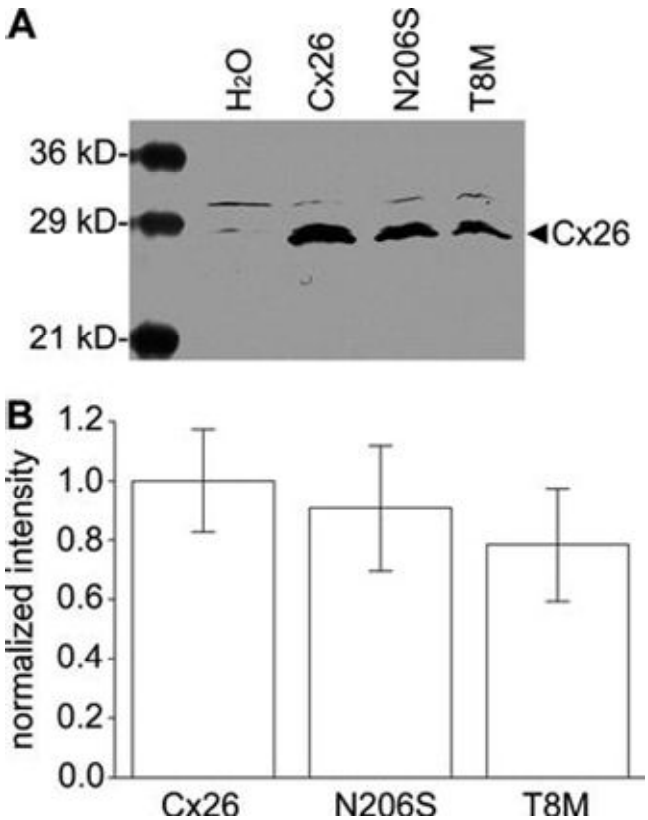

A
H2O
Cx26
N206S
T8M
36 kD
29 kD
21 kD
Cx26
B
normalized intensity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Cx26
N206S
T8M

Figure II-4. Gating properties of wild-type Cx26, T8M and N206S channels. The change in junctional currents (I_j) induced by transjunctional voltage (V_j) was plotted as a function of time for mutant and wild-type channels. **A**. For Cx26 channels, I_js decreased slowly for V_js >±80 mV. **C.** The decay in Ijs for N206S over time was slower than wild-type. **E.** In contrast, T8M has showed increased I_js for V_j <+120 mV with the exception of V_j=+120 mV, where I_j slightly decreased. For all negative V_js, the I_j increased over the course of the voltage step. **B, D, F**. The relationship of V_j to normalized steady-state conductance (G_{jss}). The smooth line shows the best fits to the Boltzmann equation and the parameters are given in Table II-1. N206S channels showed a reduced sensitivity to voltage compared with wild-type channels. T8M channels, in contrast, had characteristics markedly different from wild-type channels that could not be fit by the Boltzmann equation.

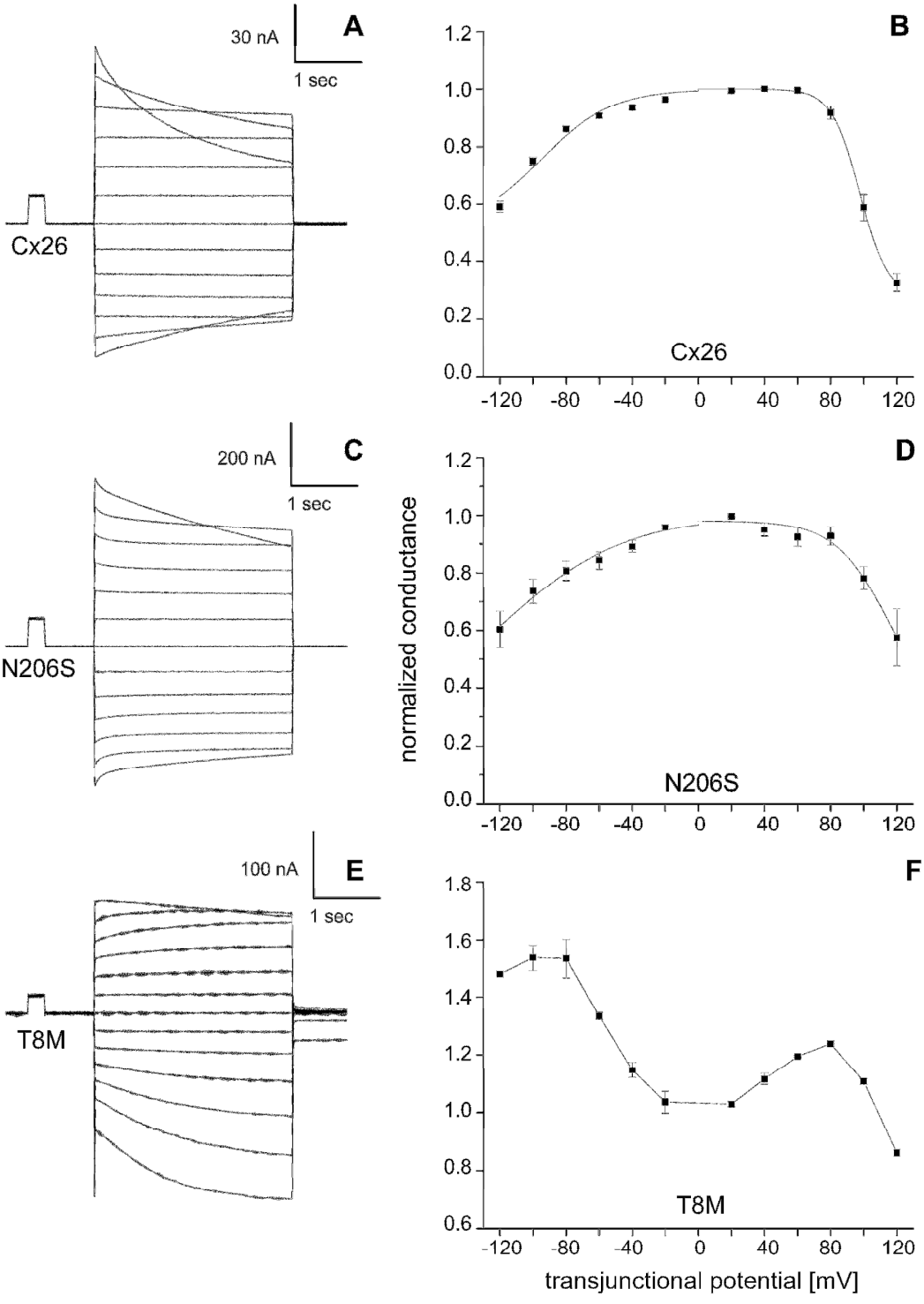

A
30 nA
1 sec
Cx26
B
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Cx26
-120
-80
-40
0
40
80
120
C
200 nA
1 sec
N206S
D
normalized conductance
N206S
E
100 nA
1 sec
T8M
F
1.8
1.6
1.4
1.2
1.0
0.8
0.6
T8M
transjunctional potential [mV]

Figure II-5. Comparative kinetic analysis of voltage-dependent channel closure for wild-type Cx26 (A) and N206S (B) channels. Representative current decays after the imposition of a Vj of +120 mV could be well fit by a monoexponential relation with the time constants (τ) indicated. The mean time channel closure constant (τ) for N206S was significantly higher than wild-type Cx26 channels.

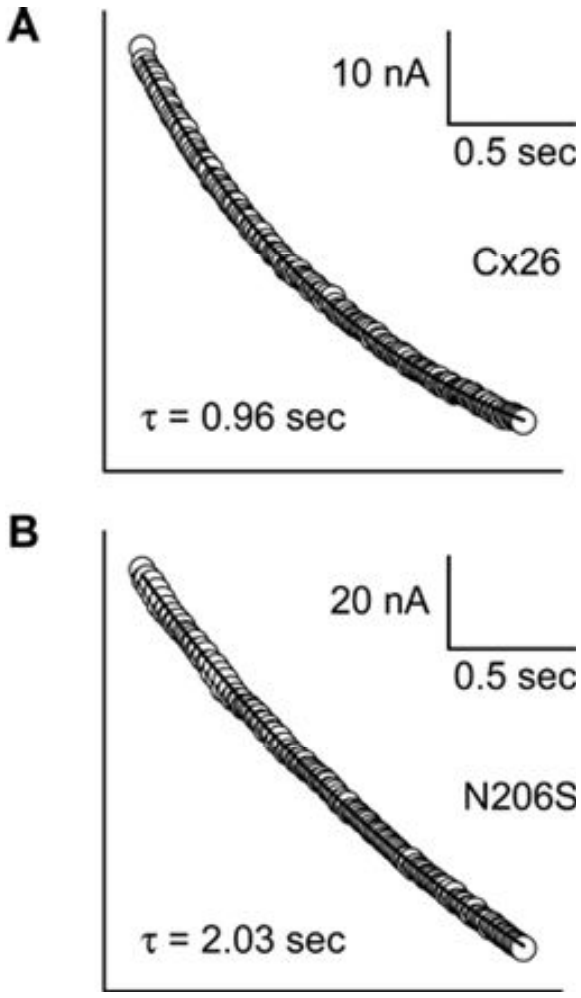

A
10 nA
0.5 sec
Cx26
τ = 0.96 sec
B
20 nA
0.5 sec
N206S
τ = 2.03 sec

Table II-1. Comparison of the Boltzmann parameters for Cx26 and N206S gap junctional channels in the Xenopus oocyte expression system. Junctional conductance (G_j) developed between pairs of Xenopus oocytes was measured by dual voltage clamp in response to increasing transjunctional potentials (V_j) of opposite polarity and normalized to the conductance measured at a V_j of ±20 mV (G_{jmax}, set as unity), as described in the Materials and Methods section. Data were fit to a Boltzmann equation of the form given in the text. G_{jmin} is the minimum conductance value as estimated from the Boltzmann fit, and V_0 is the voltage at which half-maximal decrease of G_j is measured. The cooperativity constant (A), reflecting the voltage sensitivity of the channel, reflects the equivalent number of electron charges moving through the transjunctional field. The plus and minus signs for V_j refer to the polarity of the transjunctional potential.

	V_j	V_o	G_{jmin}	A
Cx26	+	98	0.27	0.12
Cx26	-	-95	0.51	0.05
N206S	+	116	0.27	0.06
N206S	-	-123	0.20	0.03

CHAPTER III: Connexin26 Deafness Associated Mutants Show Reduced Permeability to Larger Cationic Molecules

Abstract:

Intercellular communication is important for cochlear homeostasis as connexin26 (Cx26) mutations are the leading cause of hereditary deafness. Gap junctions formed by different connexins have unique selectivity to large molecules, so compensating for the loss of one isoform can be challenging in the case of disease causing mutations. We compared the properties of Cx26 mutants T8M and N206S with wild-type channels in transfected cells using dual whole-cell voltage clamp and dye flux experiments. Wild-type and mutant channels demonstrated comparable ionic coupling and their average unitary conductance was ~106pS in 120mM K^+-aspartate$^-$ solution, documenting their equivalent K^+ permeability. Comparison of Lucifer Yellow (LY) and ethidium bromide (EtBr) transfer revealed differences in selectivity for larger anionic and cationic tracers. LY permeability to wild-type and mutant channels was similar while the transfer of EtBr through mutant channels was greatly reduced compared to wild-type junctions. Altered permeability of Cx26 to large cationic molecules suggests an essential role for biochemical coupling in cochlear homeostasis.

Introduction:

There are 21 connexin isoforms in the human genome and mutations in some of these genes have been implicated in several human hereditary diseases such as cataracts, X-linked Charcot-Marie Tooth disease, skin disorders and sensorineural hearing loss (White and Paul, 1999;Gerido and White, 2004;Rabionet et al., 2002). Gap junctions were originally believed to be non-selective for molecules smaller than 1.2 kDa (Simpson et al., 1977). Since an individual cell can express more than one isoform, it has been thought that the loss of one gene in case of mutations/deletions might be compensated for by other connexins due to their high homology. To date, gap junctions made of different connexins have been demonstrated to show little selectivity to monovalent ions, while permselectivity of each channel type to larger metabolites exhibited great variation. (Willecke et al., 1991;Nicholson et al., 2000;Valiunas et al., 2002;Harris, 2007;Goldberg et al., 2004;Weber et al., 2004).

Mutations in at least three human connexin genes, Cx26, Cx30 and Cx31, which are widely expressed throughout cochlea are the leading causes of non-syndromic hereditary hearing loss (Petit et al., 2001;Petersen and Willems, 2006). The function of gap junctional communication in the inner ear is not fully understood. However, two mechanisms have been proposed regarding the role of cochlear intercellular communication. First, it has been suggested that the gap junction network between cochlear supporting cells plays a role in the recirculation of K^+ back into the endolymph after the activation of auditory process (Kikuchi et al., 1995;Kikuchi et al., 2000b;Wangemann, 2002;Wangemann, 2006;Zhao et al., 2006). In the second model,

biochemical coupling between supporting cells during the removal of excess potassium ions around hair cells during hearing processes is thought to be important for normal cochlear function (Bruzzone and Cohen-Salmon, 2005). Analysis of one functional Cx26 recessive non-syndromic deafness mutation, V84L, demonstrated that although mutant channels were as permeable to potassium ions as wild-type junctions, the passage of IP_3 through V84L channels was impaired (Bruzzone et al., 2003;Beltramello et al., 2005), providing a support for the importance of biochemical coupling in the normal functioning of the cochlea. Further support for this view came when Ca^{2+} wave generation was compared between Cx26 wild-type and deafness-causing mutants, V84L, V95M and A88S following IP_3 injection (Zhang et al., 2005). The propidium iodide and IP_3 transfer between cells expressing mutant proteins was abolished while the mutant junctions remained permeable to Ca^{2+} and Na^{+}.

We further analyzed Cx26 recessive non-syndromic deafness mutations, the N-terminal mutant Thr8Met (T8M) and the fourth transmembrane domain mutant Asn206Ser (N206S) that were able to form functional channels in paired *Xenopus* oocytes by using dual whole-cell voltage clamp and fluorescent dye flux experiments in mammalian expression systems. We verified that these mutant proteins were expressed and correctly targeted to the cell membrane. In addition, the wild-type and mutant Cx26 channels had similar single channel characteristics and voltage-gating properties. The analysis of permeability of mutant channels demonstrated a differential selectivity of these junctions to cationic fluorescent dyes when compared to wild-type Cx26 gap junctions. The transfer of anionic Lucifer Yellow (LY) through the mutant channels was not affected, whereas their permeability to a cationic dye (ethidium bromide, EtBr) was

considerably reduced relative to wild-type Cx26. These findings support the hypothesis that Cx26 permeability to larger molecules also plays a critical role in maintaining the auditory epithelium.

Materials and Methods:

Cell Culture

Experiments were performed using intercellular communication deficient neuro-2A and HeLa cells that were individually transfected with human Cx26 wild-type, mutant T8M or N206S cDNAs. Wild-type or mutant cDNAs were subcloned into the eukaryotic expression vector pIRES2-EGFP (Clontech Laboratories, Inc, Mountain View, CA). Cells were transiently transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Briefly, one day before transfection cells were plated so that they would be 70-95% confluent the following day. Lipofectamine 2000 reagent, plasmids and OPTI-MEM medium (GIBCO) were brought to room temperature. 10μg DNA and 10μl Lipofectamine 2000 were individually diluted in 0.75ml OPTI-MEM. After 20 minutes incubation, the mix was added onto the cells drop by drop and incubated at 37^{o}C incubator for 18-24hrs. Transfection efficiency was verified by visualization of the GFP signal under a fluorescent microscope and protein expression was demonstrated by immunofluorescence staining. Transfected cells were then sub-cultured onto the micro cover glasses for electrophysiological measurements and dye flux studies within 24-48hrs after plating.

Immunofluorescent Staining

HeLa cells were grown on glass cover slips, transiently transfected with the corresponding DNAs and cultured for 24 hours. Cells were then fixed with 1% paraformaldehyde in PBS for 15min at room temperature, permeabilized with PBS/ 0.1% Triton-X 100 for 10min and then blocked with 3% BSA in PBS with 0.1% Triton-X 100 for 30min. A 1:500 dilution of a polyclonal antibody against Cx26 protein (Zymed Labs Inc, South San Francisco, CA) was applied for 1 h followed by an application of 1:2000 dilution of secondary Cy3-conjugated AffiniPure goat anti-rabbit antibody (Jackson ImmunoResearch Labs Inc, West Grove, PA) for 30 min in the dark. Before mounting, the cover slips were washed with PBS, dipped in distilled water, and mounted on slides using Vectashield with DAPI (Vector Laboratories Inc, Burlingame, CA). The protein expression and localization was monitored with 40X or 60X objectives on an Olympus BX51 microscope (Olympus America Inc., Center Valley, PA) and photographed with a MagnaFire digital camera (Optronics, Goleta, CA).

Electrophysiological Recordings

Experiments were carried out on transiently transfected N2A and HeLa cell pairs using the dual whole-cell voltage clamp method at room temperature. Cells on glass cover slips were transferred to the experimental chamber with a bath solution containing 137.7mM NaCl, 5.4mM KCL, 2.3mM NaOH, 1mM $MgCl_2$, 10mM glucose and 5mM HEPES (pH 7.4) supplemented with 2mM $CsCl_2$, 2mM $CaCl_2$ and 4mM $BaCl_2$. Patch pipettes were pulled from glass capillaries with a horizontal puller (Sutter Instruments, Novato, CA) and filled with a pipette solution of 120 mmol/L K^+ aspartate$^-$, 5mM

HEPES, 10mM EGTA and 3mM NaATP (pH 7.2) that was filtered through a 0.2µm filter. At the beginning of each experiment, both cells were clamped at the same holding potential to provide a zero transjunctional voltage. Then, one of the cells was stepped to different voltages (V_j of ±10-110mV in 20mV increments) (Valiunas et al., 2001;Brink et al., 1997). The current from the cell held at constant potential was recorded and divided by the voltage to calculate conductance. The I_js were determined at the beginning ($I_{instantaneous}$) and at the end ($I_{steady\ state}$) of each pulse. The normalized steady state conductance (g_{jss}, normalized) was then calculated by taking the ratios between I_{ss} and I_{inst}.

Fluorescent Dye Flux Experiments

Dye transfer through gap junction channels was investigated using cell pairs. Lucifer yellow (LY) and ethidium bromide (EtBr) were individually dissolved in the pipette solution at a concentration of 1mg/mL and 0.5mg/mL, respectively. One of the cells in a pair was patched with the pipette containing LY or EtBr for 12 min to allow the fluorescent dye passage from source cells to the adjacent cells. Fluorescent dye cell-to-cell spread was imaged at regular intervals using a 14-bit 16 000 pixel gray scale digital CCD-camera (HRm Axiocam, Carl Zeiss Inc, Thornwood, NY). At the end of each experiment the second cell was patched and the junctional current between the cells was measured as indicated above (Valiunas et al., 2002).

Fluorescent Data Analysis

The fluorescent intensity in the cells was directly related to either the EtBr or LY concentration. The outline of each cell and also an area of background were manually drawn in the bright field image by using AxioVision Software (Zeiss). Then, the fluorescent intensities of background, recipient cells and source cells in the defined regions were analyzed at specified time points. The averaged intensities for recipient and source cells were corrected by subtracting the background intensity from the respective images. The relative intensities were calculated at the 12min time point by the ratio of the corrected fluorescent intensities of the recipient to the source cell. Then, the relative intensities for both dyes were plotted as a function of junctional conductance and fit to a linear regression model using Spearman rank order correlation.

Signal Recording and Analysis

Patch clamp amplifiers (Axopatch 200) were used to record voltage and current signals. The current signals were digitized with a 12 bit A/D-converter (Digidata 1322A, Axon Instrument) and stored with a personal computer. Data acquisition and analysis were performed with pClamp8 software (Axon Instrument). Statistical analyses and curve fitting were performed using Origin 6.1 (OriginLab, Northampton, MA). The results are presented as mean ± SE.

Results

Protein Localization and Functional Characterization of T8M and N206S Channels in Mammalian Cells

Previously, we demonstrated that two Cx26 deafness-associated recessive missense mutations, T8M and N206S, retained some electrical coupling activity in the paired *Xenopus* oocyte expression system by dual whole-cell voltage clamp experiments (Mese et al., 2004). We wanted to further characterize the permselectivity of these mutant channels to understand how they affect the function of gap junctional communication in the inner ear. For this purpose, gap junctional communication deficient neuro-2A and HeLa cells were transiently transfected with Cx26 wild-type, T8M and N206S pIRES2-EGFP constructs. Immunofluorescent staining of transiently transfected HeLa cells verified protein expression and localization for wild-type and mutant forms of Cx26 (Figure III-1). The mutant proteins were expressed in a comparable manner to wild-type Cx26 and were properly trafficked to the cell membrane, especially at the regions of cell-to-cell contact, shown by punctate staining (arrowheads). Therefore, the expression and membrane targeting of T8M and N206S mutant proteins were not altered due to corresponding mutations when expressed in mammalian cells.

The ability of wild-type and mutant proteins to form functional channels was also analyzed by dual whole-cell patch clamp in the transiently transfected HeLa cells. We routinely treated cells with CO_2 in order to differentiate between gap junctions and cytoplasmic bridges. When intercellular currents were unresponsive to CO_2 exposure,

implying the presence of cytoplasmic bridges, those cell pairs were excluded from further data analysis. HeLa cells transfected with wild-type Cx26 formed intercellular junctions with a mean conductance of 14.6 ± 1.3 nS (n=15). The mean junctional conductance of T8M and N206S cell pairs were 12.3 ± 1.1 (n=16) and 12.8 ± 1.2 nS (n=20), respectively, values that were not statistically different from either wild-type Cx26 or each other (Figure III-2A, ANOVA, followed by Student Neuman Keuls post-hoc test, $p< 0.05$). Comparable junctional conductance between cells expressing wild-type or mutant Cx26 proteins indicated that wild-type and mutant channels had similar ionic coupling activity in mammalian cells. Cx26 wild-type channels are known to show a very weak voltage dependent response in junctional currents when expressed in mammalian cells (Valiunas et al., 1999b;Gemel et al., 2004;Yum et al., 2007). We also observed that Cx26 wild-type channels had very weak or absent voltage sensitivity and junctional currents stayed nearly constant throughout the applied voltage steps (Figure III-2B). In contrast to our previous results in paired *Xenopus* oocytes, the response of junctional currents for T8M and N206S channels to a range of applied voltages was similar to wild-type Cx26 in mammalian cells, exhibiting little decay during the voltage steps (Figure III-2B).

Single Channel Properties of Wild-Type and Mutant Proteins

Following verification of proper junction formation for the mutant channels, we examined single channel events to look for differences in the unitary conductance or gating between the mutant and wild-type connexins. In poorly coupled Neuro-2A cell pairs, individual single channel events were recorded using dual whole-cell voltage

clamp. Representative current traces recorded at ±110mV in 120mM potassium aspartate pipette solution for Cx26 wild-type, T8M and N206S single channels are illustrated in Figure III-3A. The magnitude of unitary current change in these recordings represents K^+ permeability through the corresponding single channels. Comparable changes in the traces of wild-type (110pS and 98pS for positive and negative voltages, respectively), T8M (123pS and 106pS) and N206S (108pS) channels implicated a similar potassium transfer between the cells. In addition, these channels spent long periods of time in the fully open state at a transjunctional voltage (V_j) of ±110 mV which correlates with the weak voltage-dependent response seen in macroscopic recordings. The averaged unitary conductance of wild-type channels was 105±2.9 pS (n=51) in 120 mM K^+-aspartate$^-$ pipette solution which was consistent with previous reports (Valiunas et al., 1999b;Beltramello et al., 2005;Bicego et al., 2006;Hernandez et al., 2007). The unitary conductance of T8M (n=24) and N206S (n=60) were 106±4.1pS and 107±2.2pS, respectively (Figure III-3B). Comparable unitary conductance between wild-type and the mutant channels ($p< 0.05$, ANOVA, followed by Student Neuman Keuls post-hoc test) demonstrated that T8M and N206S junctions were as permeable to K^+ as wild-type Cx26. Thus, the mutations did not affect the permeability of potassium ions transferred between the cells. In addition, since the average macroscopic conductance and the average unitary conductance were not statistically different, these data show that an equal number of operational channels (~125) were present between the paired cells in each tested condition.

The Permeability of Wild-Type and Mutant Channels to Larger Molecules

Following the observation that Cx26 wild-type and mutant channels were equally permeable to K^+, we next wanted to look for possible differences in their selectivity to larger molecules. For this, we used fluorescent dyes with different molecular weights and charges since they are not rapidly metabolized like biological molecules, and they can be directly quantified by epifluorescence. We first compared the permeation of an anionic dye, Lucifer Yellow (LY, MW 457; charge -2) through mutant and wild-type channels using dye flux experiments followed by measurements of junctional conductance (g_j) in individual cell pairs (Valiunas et al., 2002). Figure 4A illustrates cell pairs expressing wild-type and mutant T8M and N206S channels as an example of LY passage at comparable macroscopic junctional conductance (22-26 nS). The fluorescent intensity in both recipient and source cells was monitored at regular intervals after the patch seal of the source cell was opened (Figure III-4A; 2 min, 5 min and 12 min are shown). The transfer of LY through wild-type, T8M and N206S channels could be observed by the increase in the fluorescent intensity in the recipient cells for all samples. Fluorescent values for source and recipient cells were normalized to the maximum intensities in the corresponding pairs and then plotted as a function of time. The source cells expressing wild-type, T8M and N206S channels had similar loading patterns and stayed constant after reaching the saturation (Figure III-4B). However, there was a time dependent increase in the fluorescent intensities of all recipient cells over 12 min (Figure III-4C). The normalized values in the recipient cells of wild-type, T8M and N206S were not statistically different from each other ($p<0.05$, ANOVA). The increase in the fluorescent intensity in the recipient cells could be fitted by linear regression with similar slopes for

wild-type, T8M and N206S channels (R^2 values of 0.95, 0.99 and 0.98, respectively, Figure III-4C).

After verification of comparable LY dye transfer between the cell pairs expressing the wild-type and mutant channels, we next studied the flux of a positively charge molecule, ethidium bromide (EtBr; MW 394; charge +1). Figure III-5A illustrates examples of cells tested for EtBr transfer between pairs with similar junctional conductance (14-15 nS). The fluorescent intensity in the recipient cell transfected with wild-type Cx26 increased over time and dye flux from the source cell to the recipient cell was evident starting from 5 min after opening the seal. On the other hand, there was little increase in the fluorescent intensity of recipient cells for T8M and N206S channels even at the 12 min time point (Figure III-5A). The normalized intensities versus time plots for the source cells of the examples shown in figure IV-5A revealed that there was some individual variation in the rate of cell loading, but all source cells reached a maximum intensity by 12 minutes (Figure III-5B). Furthermore, the mean time course of source cell loading was not statistically significant between wild-type and mutant experiments (Table III-1, see below). The normalized intensities for recipient cells of T8M and N206S showed only a very small increase over 12 min (R^2 values for the linear regression were 0.99 for both, Figure III-5C). The EtBr fluorescent intensity increase in the recipient cells expressing wild-type Cx26 channels (the R^2 value of linear regression was 0.98, with a ~3 fold increase in the slope) was much higher than that seen for mutant T8M and N206S channels. In dye permeability experiments, the diffusion of tracer into the source cells from the patch pipettes is the rate limiting step (Oliva et al., 1988;Mathias et al., 1990) . We determined the time necessary for source cells of wild-type, T8M and N206S to reach

steady state intercellular concentrations for both LY and EtBr. The averaged steady state time for wild-type, T8M and N206S was 183±50, 185±29 and 197±17 sec for LY experiments and 311±53, 329±32 and 332±31 sec for EtBr dye flux (Table III-1). The time constants for wild-type, T8M and N206S in either LY or EtBr permeability experiments were not statistically different from each other ($p<0.05$, ANOVA). Taken together, these data show that the observed reduction in the permeability of mutant channels to EtBr were not due to variations in the loading of the source cell with dye, or differences in the numbers of operational channels, but resulted from intrinsic alterations of channel permeability to EtBr.

Measurements of LY or EtBr dye flux and junctional conductance (g_j) in many cell pairs with different magnitudes of total conductance enabled further comparison of dye transfer among wild-type, T8M and N206S channels (Figure III-6). If the dye concentration in the source cell is much greater than that of the recipient cell and the channels have equivalent unitary conductance, then the amount of dye flux in a fixed time would be expected to rise linearly as channel number increases. We observed a linear correlation between the junctional conductance and the relative intensity of the recipient cells expressing wild-type channels in LY transfer experiments (R^2 for linear regression was 0.90), that is, as the junctional conductance increased, more LY flux was observed at the end of the 12 minute time period analyzed (Figure III-6A). A linear relation was also noticed for the mutant channels, where as the number of channels increased so did the amount of LY passing through channels (R^2 values of T8M and N206S were 0.96 and 0.92, respectively). Comparison of LY transfer among all channel types revealed that the T8M and N206S mutations did not affect Cx26 channel

permeability to LY since the LY flux through mutant channels was indistinguishable from wild-type junctions ($p<0.05$, ANOVA, Figure III-6A). There was also a linear correlation between the junctional conductance and relative fluorescent intensity of recipient cells expressing wild-type Cx26 for EtBr flux (R^2 value of 0.92, Figure III-6B). In contrast, mutant channels were poorly fit by linear regression (R^2 values were 0.48 for T8M and 0.55 for N206S), likely due to their poor permeability to EtBr, which was greatly decreased relative to wild-type Cx26. There were 72.4% and 72.0% reductions in the apparent slopes of fitted lines of T8M and N206S, respectively (Figure III-6B). Thus, while T8M and N206S mutant channels passed negatively charged LY as well as wild-type junctions, their permeability to cationic EtBr was much lower.

Discussion:

Functional characterization of connexin26 missense mutations associated with non-syndromic hearing loss may provide insights about the role of gap junctional communication in the inner ear. To date, most of the analyzed Cx26 mutations were not able to form functional channels (Palmada et al., 2006;Melchionda et al., 2005;Beltramello et al., 2005;Bruzzone et al., 2003). However, a small subset of mutants has been shown to retain some level of ionic coupling (Palmada et al., 2006;Zhang et al., 2005;Bruzzone et al., 2003). These partially active mutants might help to characterize the type of molecules needed to be exchanged between cochlear supporting cells to maintain normal hearing. Here, we analyzed two partially functional Cx26 deafness associated missense mutations, T8M and N206S. We first verified the expression and proper membrane targeting of mutant channels. Then, we demonstrated the ability of mutants to form functional channels in a mammalian expression system with a total junctional conductance comparable to wild-type Cx26. In addition, the wild-type and mutant channels were shown to have similar unitary conductance when measured with a 120mM K^+ pipette solution, implicating that mutant junctions were as permeable to potassium ions as wild-type Cx26. Examination of their permeability to larger molecules revealed a differential selectivity of mutant channels for fluorescent dyes with different molecular weight and charge. Although Lucifer yellow transfer through the mutant junctions was comparable to wild-type channels, their permeability to ethidium bromide was greatly reduced (summarized in Table III-1). These observations provide support for the

contribution of biochemical coupling within the supporting cell gap junction networks in the maintenance of cochlear homeostasis.

Cx26 wild-type channels are known to display weak voltage sensitivity in paired *Xenopus* oocytes where they have shown asymmetric junctional current decays during application of high voltages (higher than ±80mV) (Barrio et al., 1991;Verselis et al., 1994;Skerrett et al., 2004;Beahm et al., 2006). On the other hand, when expressed in mammalian cells, they often appear to lose this weak voltage dependence (Gemel et al., 2004;Yum et al., 2007). Here, we also report that Cx26 wild-type channels had no apparent voltage dependent changes in their junctional currents and the mutant channels had similar responses to the applied voltages. It is not clear why Cx26 channels show different macroscopic gating characteristics in these two model systems.

At least four connexin isoforms, Cx26, Cx30, Cx31 and Cx43, have been shown to be expressed in the inner ear (Kikuchi et al., 1995;Lautermann et al., 1998;Cohen-Salmon et al., 2004;Forge et al., 2003). Cx26 and Cx30 are the main components of gap junction channels between the cochlear supporting cells where they were shown to be found in the same gap junction plaques. The ability of Cx26 and Cx30 to form heteromeric and/or heterotypic channels has been demonstrated by several labs (Zhao and Yu, 2006;Ahmad et al., 2007;Yum et al., 2007). Cx26 and Cx30 share the highest homology among the members of connexin gene family. Therefore, it has been speculated that the loss of either gene in the inner ear due to mutations might be compensated for by the intact isoform (Ahmad et al., 2007). However, other studies have suggested that this may not always be the case. For example, Cx30 channels were shown to be more permeable to cationic dyes than to anionic ones (Beltramello et al., 2003;Sun

et al., 2005). On the other hand, Cx26 channels were demonstrated to be permeable to both cationic and anionic dyes and they were suggested to be primarily responsible for the permeability of anionic molecules in the inner ear (Nicholson et al., 2000;Beltramello et al., 2003;Zhao, 2005). Here, we demonstrated that Cx26 mutant T8M and N206S channels had impaired permeability to a cationic dye, ethidium bromide, while they retained their ability to transfer the anionic Lucifer Yellow between cell pairs. Previously, Zhang et al. showed that Cx26 mutations V84L, V95M and A88S also lost their permeability to another cationic dye, propidium iodide (Zhang et al., 2005). The altered permeability of Cx26 channels to positively charged molecules could help explain why these mutants generate deafness, despite remaining permeable to K^+. For example, they may result in an overloading of Cx30 channels with regard to endogenous cationic molecule transfer, rendering Cx30 unable to compensate for the partial loss of Cx26 function in deaf patients.

The role of intercellular communication in the inner ear is not fully understood. However, two hypotheses have been proposed. The first emphasizes the importance of ionic coupling between supporting cells where cochlear gap junctions play a role in the recirculation of K^+ (Kikuchi et al., 2000b). The second model suggests a more local mechanism that included permeability of larger solutes such as IP_3 (Bruzzone and Cohen-Salmon, 2005). Partially functional mutants might provide an invaluable tool for differentiating the role of ionic coupling from biochemical coupling through the gap junctional networks in the cochlea. The unitary conductance of Cx26 wild-type, T8M and N206S channels were statistically indistinguishable from each other. This implicated that all channel types had the same K^+ permeability. However, they showed differential

selectivity to larger molecules. Hence, deafness associated with missense mutations in Cx26 cannot only be due to disruption of potassium permeability, rather abnormalities in the transfer of large molecules between the supporting cells must also play a role. Our data support the importance of biochemical coupling for normal cochlear functioning. Generation of mouse models using these Cx26 mutant variants might improve our understanding of their *in vivo* effect on intercellular communication and the molecules needed to be exchanged between the supporting cells during normal hearing.

Figure Legends and Figures:

Figure III-1. Cx26 wild-type and mutant T8M and N206S protein expression and localization. HeLa cells were transiently transfected with wild-type, T8M and N206S constructs and were examined by immunostaining and fluorescence microscopy. Cells individually expressed wild-type and mutant T8M and N206S proteins and properly targeted them to the cell membrane especially at the region of cell-to-cell apposition. Arrowheads point to the punctate staining of Cx26 gap junction plaques at cell-cell contact areas. Green represents GFP, blue shows DAPI staining of cell nuclei and red is Cy3 staining of Cx26 protein. Scale bar = 10 μm

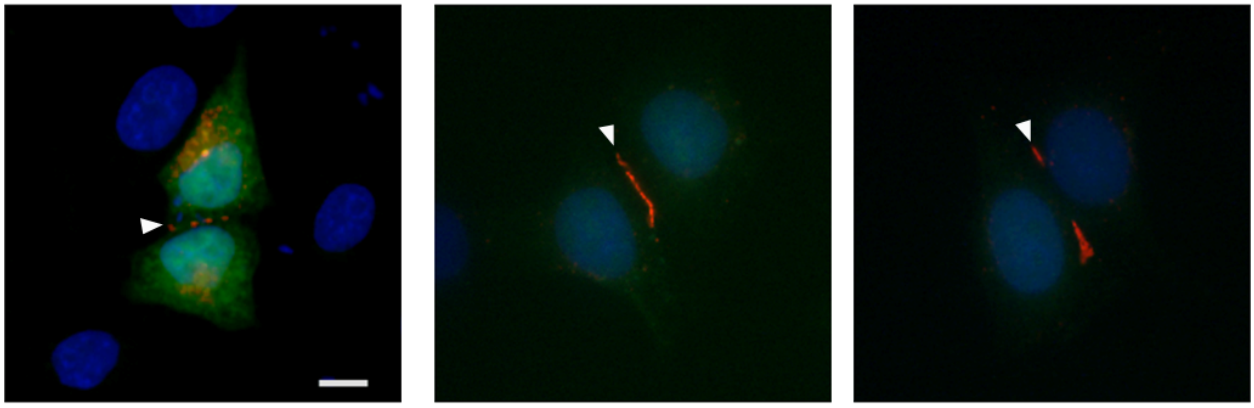
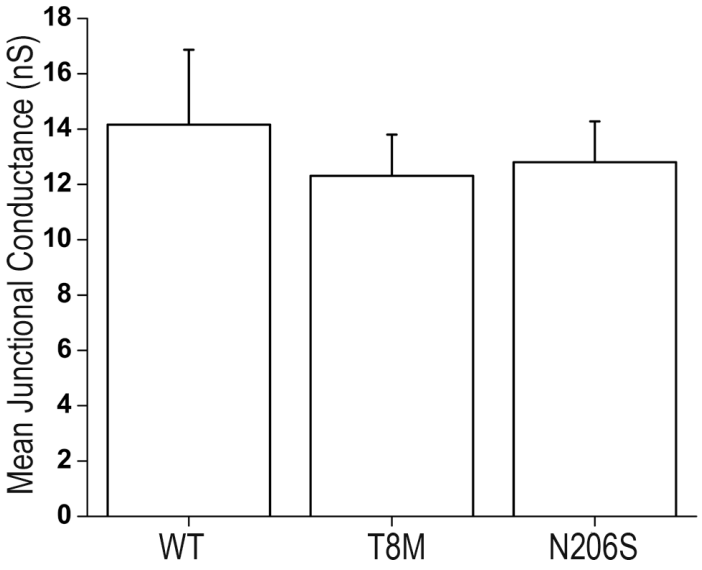

WT
T8M
N206S

Figure III-2. Conductance and voltage gating properties of wild-type, T8M and N206S channels. A. Comparison of macroscopic junctional conductance of Cx26 wild-type (n=15), T8M (n=16) and N206S channels (n=20) in transiently transfected HeLa cells showed that they were not statistically different from each other (ANOVA, $p<0.05$). Data are the mean ± S.E. **B.** (Left panel) Junctional currents from HeLa cell pairs transfected with wild-type Cx26, T8M and N206S were recorded during application of a series of transjunctional voltages (V_js) ranging from +110 to –110 mV in 20-mV increments. (Right panel) The normalized junctional conductance (mean ± S.E.) versus transjunctional voltages was plotted. Cx26 wild-type and mutant T8M and N206S channels demonstrated very little voltage dependence.

A


Mean Junctional Conductance (nS)
18
16
14
12
10
8
6
4
2
0
WT
T8M
N206S

B

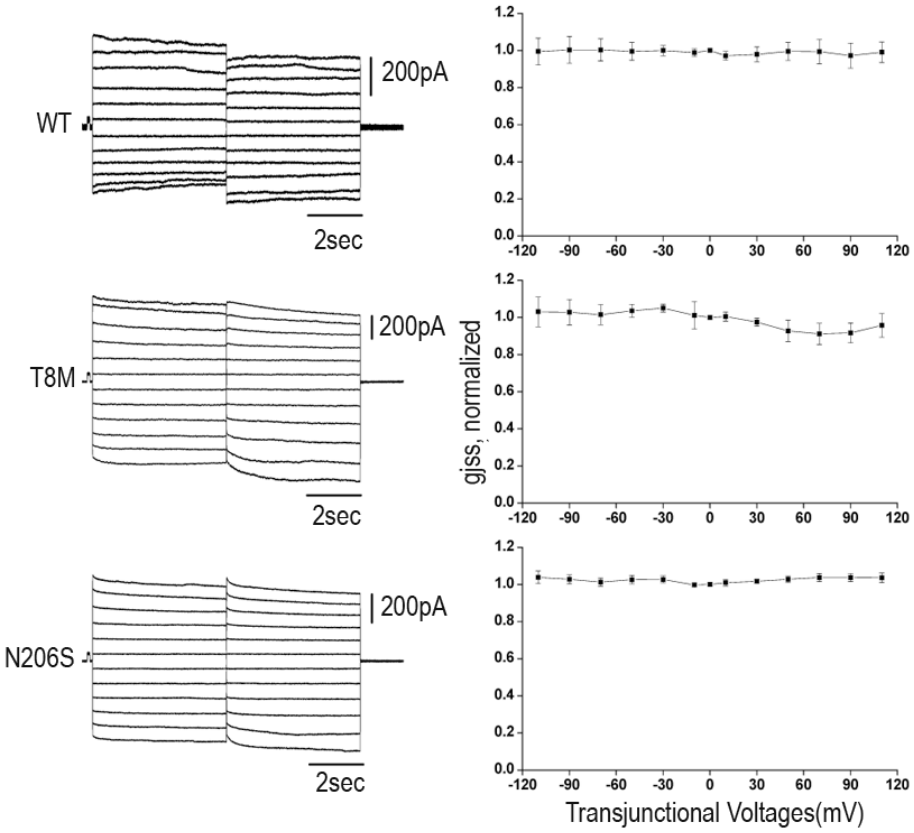
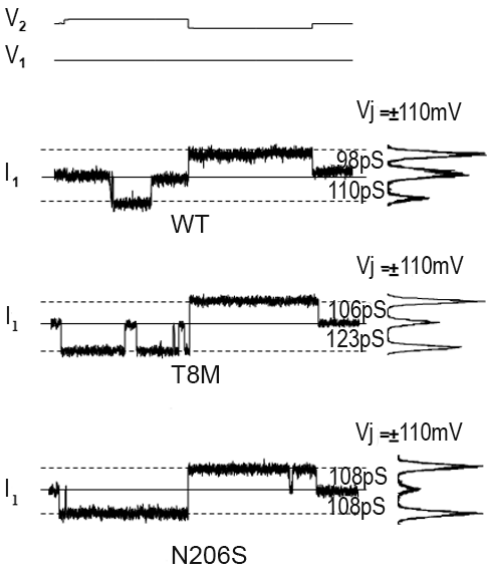

WT
200pA
2sec
T8M
200pA
2sec
N206S
200pA
2sec
gjss, normalized
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-120
-90
-60
-30
0
30
60
90
120
Transjunctional Voltages(mV)

Figure III-3. Single channel properties of wild-type, T8M and N206S channels. Single channel currents recorded from cell pairs expressing wild-type, T8M and N206S at a V_j of ±110mV in transiently transfected Neuro-2A cells. **A.** The current histograms of the single channels for wild-type and mutant connexins revealed unitary conductances between 98pS and 123pS. The solid line represents the zero junctional current and the dashed lines indicate open state currents. **B.** The averaged unitary conductances of wild-type (n=51), T8M (n=24) and N206S (n=60) channels were statistically indistinguishable from each other (ANOVA, $p< 0.05$). Data represented as mean ± S.E.

A **B**


V2
V1
Vj =±110mV
I1
98pS
110pS
WT
Vj =±110mV
I1
106pS
123pS
T8M
Vj =±110mV
I1
108pS
108pS
N206S

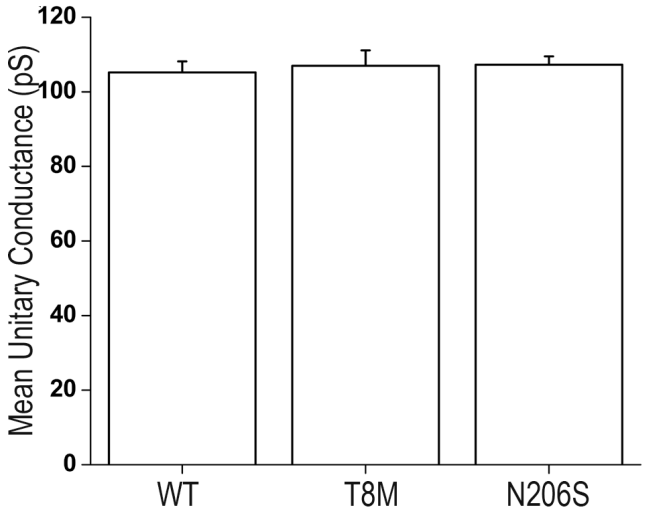
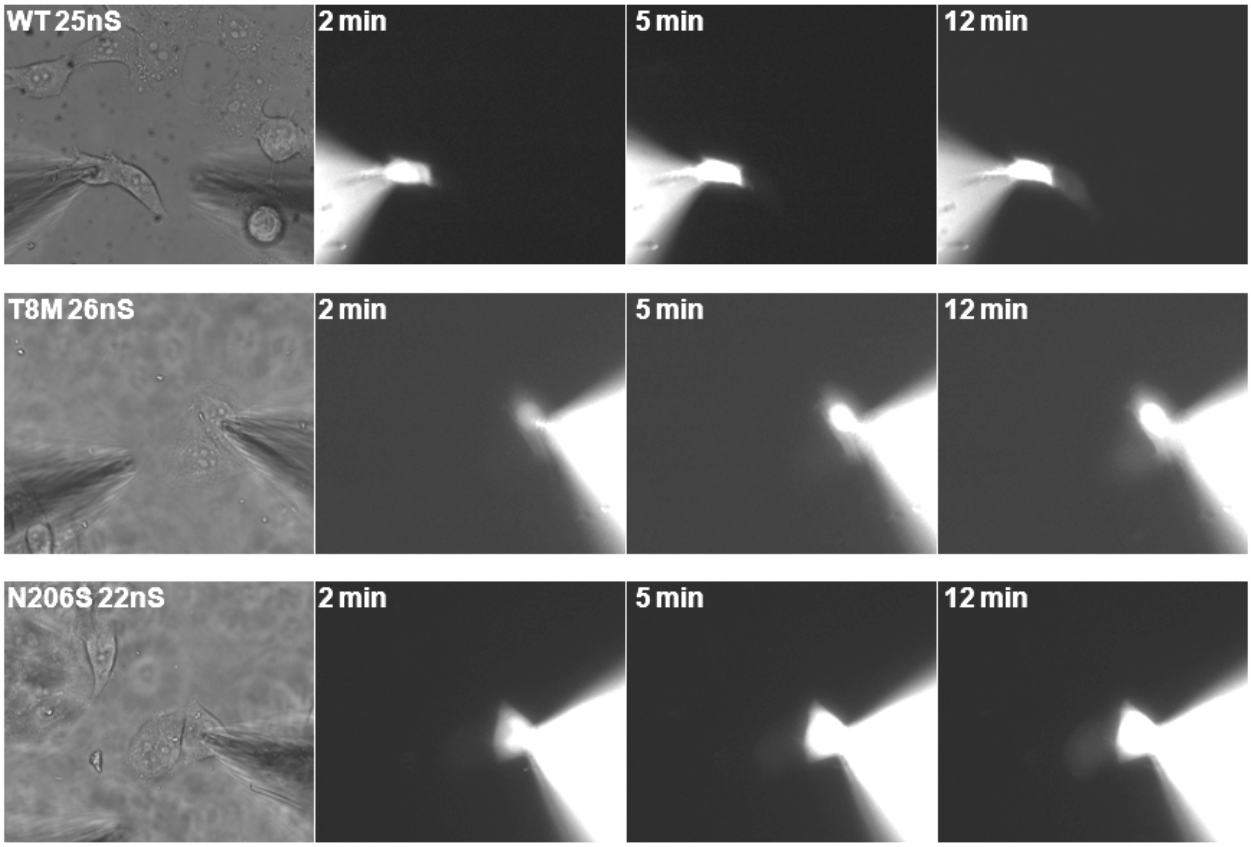
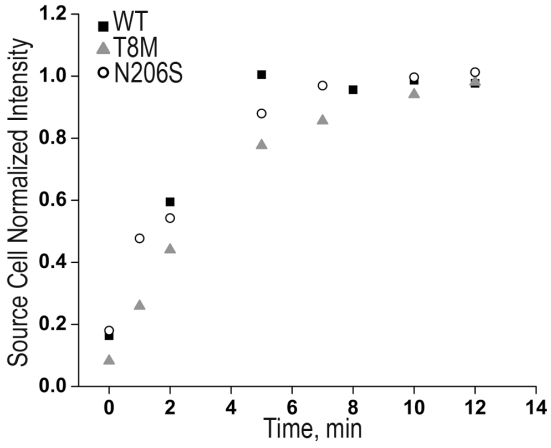

Mean Unitary Conductance (pS)
120
100
80
60
40
20
0
WT
T8M
N206S

Figure III-4. Permeability of wild-type and mutant channels to Lucifer yellow (LY). Measurements of LY flux from cell pairs expressing wild-type Cx26, T8M and N206S followed by measurements of junctional conductance (g_j). **A.** Epifluorescent micrographs taken at 2, 5 and 12min for cell pairs of similar junctional conductance (22-26nS) demonstrated progressive fluorescent intensity increases in the recipient cells for each channel type. **B**. The fluorescent intensities of the source cells of wild-type (■), T8M (▲) and N206S (○) were normalized to the maximum fluorescent value in the source cells and was plotted as a function of time. For all three examples shown in A, the source cells had similar loading patterns. **C.** The normalized intensities of recipient cells expressing wild-type (■), T8M (▲) and N206S (○) showed a time dependent increase in the intensities of recipient cells over the course of 12min, implicating dye transfer from the source cells to the recipient cells.

A


WT 25nS
2 min
5 min
12 min
T8M 26nS
2 min
5 min
12 min
N206S 22nS
2 min
5 min
12 min

B


WT
T8M
N206S
Source Cell Normalized Intensity
Time, min
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0
2
4
6
8
10
12
14

C

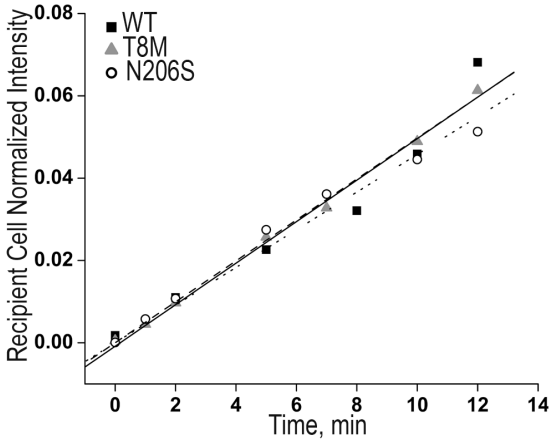
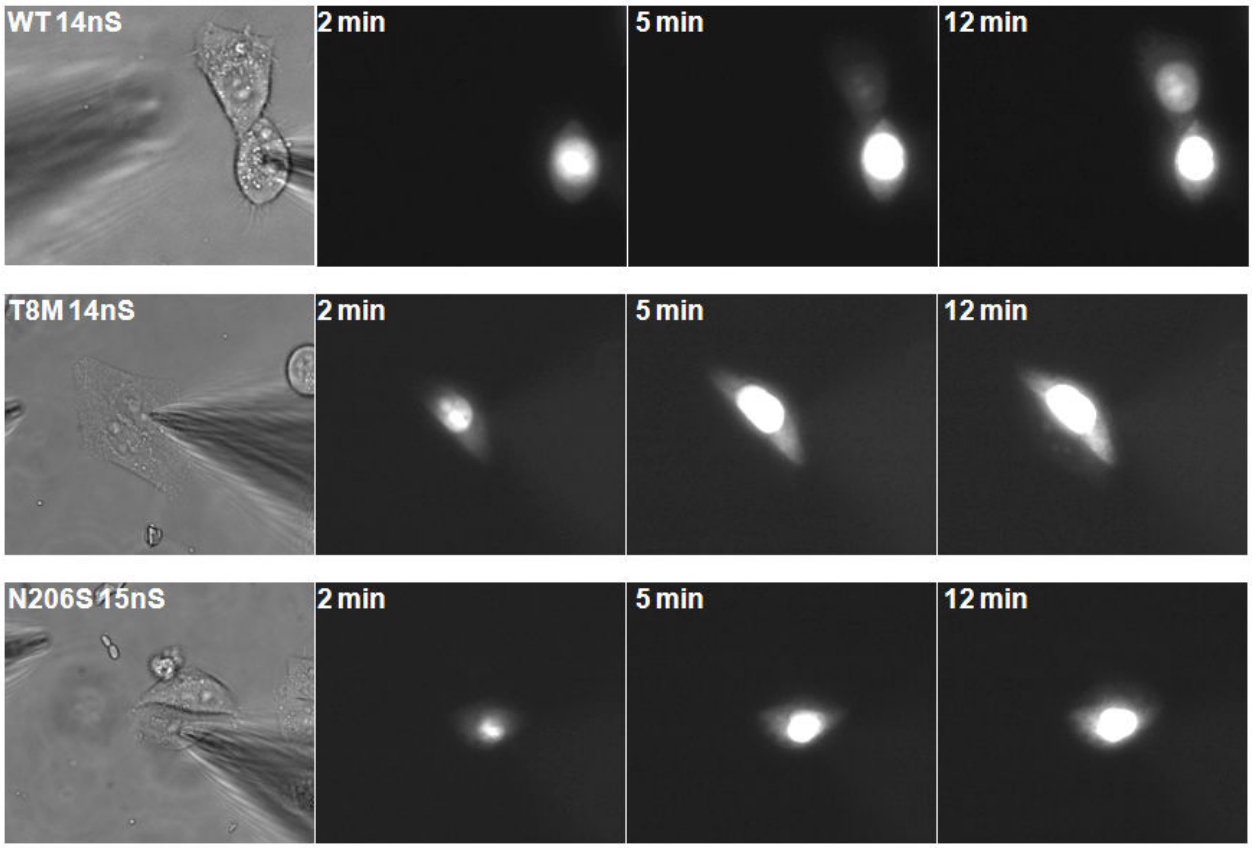
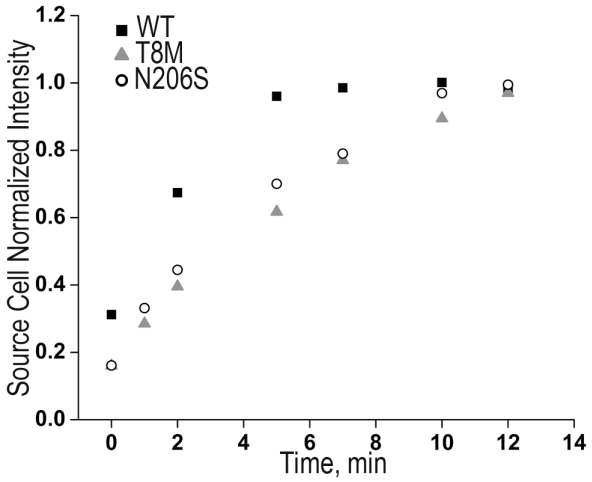

WT
T8M
N206S
Recipient Cell Normalized Intensity
Time, min
0.00
0.02
0.04
0.06
0.08
0
2
4
6
8
10
12
14

Figure III-5. Permeability of wild-type and mutant channels to ethidium bromide (EtBr). EtBr transfer through cells expressing the wild-type and mutant channels was compared between pairs with similar junctional conductance. **A.** Examples of epifluorescent micrographs (taken at 2, 5 and 12min) of cell pairs expressing wild-type, T8M and N206S proteins during EtBr flux experiments. Transfer of EtBr through wild-type Cx26 channels was readily observed after the opening of the patch seal in the source cell, as seen by the increased intensity of the recipient cell. There was little visible fluorescent intensity in the recipient cells coupled by T8M and N206S channels. **B**. Normalized fluorescent intensities of source cells expressing wild-type (■), T8M (▲) and N206S (○) Cx26 in figure A were plotted as a function of time (min) to show dye injection into the cells. **C.** The fluorescent intensity of the recipient cells was increased over time. Both the T8M (▲) and N206S (○) mutant channels had greatly reduced permeability relative to wild-type channels (■).

A


WT 14nS
2 min
5 min
12 min
T8M 14nS
2 min
5 min
12 min
N206S 15nS
2 min
5 min
12 min

B


WT
T8M
N206S
Source Cell Normalized Intensity
Time, min
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0
2
4
6
8
10
12
14

C

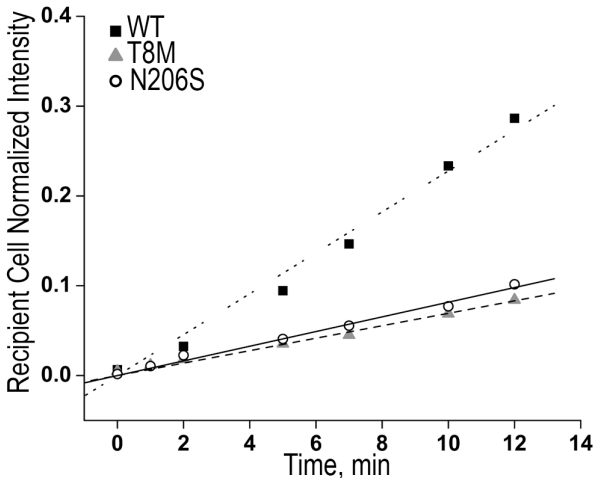

WT
T8M
N206S
Recipient Cell Normalized Intensity
Time, min
0.0
0.1
0.2
0.3
0.4
0
2
4
6
8
10
12
14

Figure III-6. The relative intensity of recipient cells for LY and EtBr as a function of junctional conductance. The relative intensities of recipient cells of Cx26 wild-type (■), T8M (▲) and N206S (○) junctions in different cell pairs were analyzed at the 12 min time point and plotted against the junctional conductance (g_j). **A.** The relationship between the relative intensity and the junctional conductance for wild-type, T8M and N206S for LY was linear (R^2 values were 0.90 (straight line), 0.96 (dashed line) and 0.92 (dotted line), respectively). LY passage between pairs of cells expressing wild-type, T8M and N206S was comparable to each other, and statistically were not different (ANOVA, $p<0.05$). **B.** EtBr transfer between cell pairs expressing wild-type Cx26 showed a modest linear correlation between relative intensity of recipient cell and junctional conductance with a R^2 value of 0.92 (straight line). T8M and N206S channels showed only a weak correlation between juctional conductance versus relative intensity of recipient cell and greatly reduced EtBr permeability. The slopes of fitted lines for T8M (dashed fitting line) and N206S (dotted fitting line) channels were 27.6% and 28% of that of wild-type Cx26.

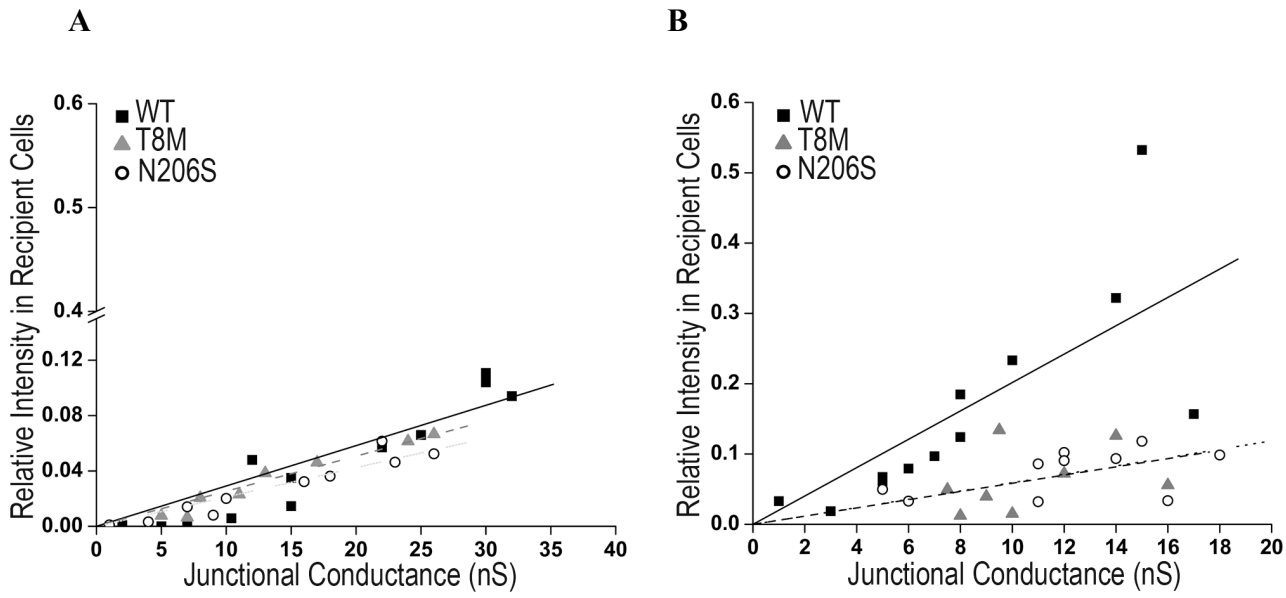

A
B
WT
T8M
N206S
Relative Intensity in Recipient Cells
Junctional Conductance (nS)
0.6
0.5
0.4
0.12
0.08
0.04
0.00
0
5
10
15
20
25
30
35
40
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
2
4
6
8
10
12
14
16
18
20

Table III-1: Comparison of properties of Cx26 wild-type, T8M and N206S channels. Values are mean± S.E. [a] n is number of cell pairs and [b] n is number of events. The apparent slopes of the fitted data for relative intensity of recipient cell versus junctional conductance in many cell pairs are presented for LY and EtBr flux experiments. The time constants for source cells to reach steady state were determined for wild-type, T8M and N206S in both LY and EtBr permeability experiments and revealed that the source cell loadings were similar to each other ($p<0.05$, ANOVA).

Channel Type	Mean Junctional Conductance (nS)	Mean Unitary Conductance (pS)	Apparent Slope of LY Transfer	Apparent Slope of EtBr Transfer	Source Cell Steady State Loading Time (sec), LY	Source Cell Steady State Loading Time (sec), EtBr
WT	14.6 ± 1.3 (n=15[a])	105 ± 2.9 (n=51[b])	0.0029	0.021	183 ± 50 (n=7[a])	311 ± 53 (n=16[a])
T8M	12.3 ± 1.1 (n=16[a])	106 ± 4.1 (n=24[b])	0.0025	0.0058	185 ± 29 (n=7[a])	329 ± 32 (n=6[a])
N206S	12.8 ± 1.2 (n=20[a])	107 ± 2.2 (n=60[b])	0.0021	0.0059	197 ± 17 (n=9[a])	332 ± 31 (n=7[a])

Chapter IV: Concluding Remarks and Future Directions

Gap junction channels play a role in many cellular events by facilitating the electrical and/or metabolic coupling of adjacent cells. The identification of connexin proteins as the primary component of intercellular channels provided a hallmark in the field after which a great deal of progress has been made to understand the properties of gap junction channels as well as their physiological roles in a variety of tissues (Goodenough, 1974;Stevenson and Paul, 1989;Harris, 2001;White and Paul, 1999). The connexin gene family consists of 20 members in the mouse and 21 members in the human genome (Willecke et al., 2002;Sohl et al., 2004), which share structural similarities. Gap junction channels were initially anticipated to be non-selective pores which would allow the free passage of molecules smaller than 1.2 kDa. However, it is now well documented that the composition of channel affects its permeation to larger molecules (Bedner et al., 2006;Niessen et al., 2000;Cao et al., 1998;Weber et al., 2004;Nicholson et al., 2000;Alexander et al., 2004;Goldberg et al., 2002;Goldberg et al., 1999).

There are over 50 missense mutations throughout human Cx26 gene that are associated with non-syndromic recessive deafness. Characterization of the mutant channels provided an understanding about the effect of these mutations on channel activity, and also a tool for the identification of the types of molecules which need to be exchanged between the supporting cells. Many of the analyzed missense mutations were not able to form functional channels, so these mutations mostly provided information about the role of each residue on connexin and/or channel biosynthesis. On the other

hand, a small fraction of Cx26 missense mutations was shown to retain some level of activity. Examination of these partially functional mutations helped to distinguish the role of ionic coupling from the biochemical coupling in the cochlear normal functioning.

In this dissertation, five Cx26 deafness associated mutations, T8M, R143W, V153I, N206S and L214P were tested to understand their mode of action in the generation of deafness. R143W, V153I and L214P mutants were not able to form functional channels. The complete loss of Cx26 mediated intercellular communication between the supporting cells due to these mutations might explain deafness in these individuals. Nevertheless, the level at which these mutations lack channel activity still remains to be established. Since the gap junction channel structure at the atomic level has not been resolved, it is hard to predict how each mutation affects the channel pore. Two mutants, T8M and N206S, facilitated the channel formation, but with different gating properties than wild-type channels.

Connexins show differential selectivities in their permeabilities to second messengers and large metabolites. The factors that facilitate the channel permeability are not known. It has been suggested that factors such as size, charge, shape of molecules or affinity between the molecule and the pore either individually or in might determine channel permselectivity (Goldberg et al., 1999;Nicholson et al., 2000;Weber et al., 2004). Furthermore, the affinity between the biological molecules and the channel has also been suggested to play role that influences the selectivity of connexins (Weber et al., 2004;Harris, 2007;Ayad et al., 2006). The properties of T8M and N206S channels were further analyzed for characterization of their mode of action on channel function and their presumed effect on the cochlear supporting cell gap junction network. These channels

produced junctional conductances comparable to wild-type Cx26 channels, and they were as permeable to potassium ions as wild-type junctions in mammalian cells. When the transfer of larger molecules through the channels was compared with wild-type Cx26, they exhibited differential properties in their permeability. These mutant channels remained permeable to an anionic fluorescent dye, Lucifer Yellow (MW 457 Da; charge -2) whereas they had greatly diminished flux of a cationic dye, ethidium bromide (MW 394; charge +1) from the source cells into the recipient cells. The deafness associated with these mutations therefore was not due to loss of K^+ re-circulation pathways since these channels had comparable potassium ion permeability as wild-type Cx26. Alterations in the channel permeability to larger molecules supported that biochemical coupling between the Cx26 junctions plays an important role in the cochlear physiology. The usage of different dye molecules for the analysis of mutant channel permeability suggested that molecular weight was not a major determinant for Cx26 channels, since the mutant channels remained permeable to a larger molecule (LY) than ethidium bromide. The charge of the molecule, its structure and/or the molecular dimensions might play a role in the determination of LY and EtBr permeation. The minimal and maximal dimensions for LY are 9.5 and 10.6 Å with a ratio of 1.1 while those for EtBr are 9.3 and 11.6 Å with a 1.3 ratio, respectively. Furthermore, these mutations may affect the affinity of channels to EtBr and hence altering its permeability. Still, it is difficult to predict how the mutations in the Thr8 and Asn206 residues interfere with the channel structure and alter its permselectivity.

Further characterization of these partially active mutants is required to understand the function of gap junctional communication in the inner ear to identify cytoplasmic

compounds needed to be transferred between the supporting cells. Cx26 homomeric channels were shown to be permeable to both IP_3 and cAMP (Hernandez et al., 2007;Harris, 2007). The transfer of these biological molecules through the mutant channels can be tested to determine if the mutations alter channel permeation to these metabolites. Such *in vitro* characterization gives only possible effects of these mutations on channel function. For the assessment of the *in vivo* physiological effect on cochlear homeostasis, generation of animal models that carry the partially functional Cx26 mutations (knock-in animals) will be needed. The functional, anatomical and histological comparisons of Cx26 knock-in and wild-type animals can verify the relevance of the *in vitro* observations for the function of Cx26 in the cochlea.

In conclusion, this dissertation demonstrated that a subset of Cx26 deafness associated missense mutations resulted in the disruption of biochemical coupling between the transfected cells while still remained permeable to K^+. This provides support for the importance of metabolic coupling mediated by Cx26 channels in the inner ear and suggests that the disruption of exchange of larger molecules through Cx26 due to mutations plays a role in alteration of the cochlear homeostasis and results in the generation of hearing loss.

CHAPTER V: References

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