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Developmental Expression of *Kcnq4* in Vestibular Neurons and Neurosensory Epithelia

Sonia M.S. Rocha-Sanchez^{1,2,*}, Kenneth A. Morris², Bechara Kachar³, David Nichols², Bernd Fritzsche², and Kirk W. Beisel²

¹ Department of Oral Biology, Creighton University School of Dentistry, 2500 California Plaza, Omaha, NE 68178, USA

² Department of Biomedical Sciences, Creighton University, 2500 California Plaza, Omaha, NE 68178, USA

³ Structural Cell Biology section, laboratory of Cell Biology, NICD/NIH, Bethesda, MD 20892, USA

Abstract

Sensory signal transduction of the inner ear afferent neurons and hair cells (HCs) requires numerous ionic conductances. The KCNQ4 voltage-gated M-type potassium channel is thought to set the resting membrane potential in cochlear HCs. Here we describe the spatiotemporal expression patterns of *Kcnq4* and the associated alternative splice forms in the HCs of vestibular labyrinth. Whole mount immunodetection, qualitative and quantitative RT-PCR were performed to characterize the expression patterns of *Kcnq4* transcripts and proteins. A topographical expression and upregulation of *Kcnq4* during development was observed and indicated that *Kcnq4* is not restricted to either a specific vestibular structure or cell type, but is present in afferent calyces, vestibular ganglion neurons, and both type I and type II HCs. Of the four alternative splice variants, *Kcnq4_v1* transcripts were the predominant form in the HCs, while *Kcnq4_v3* was the major variant in the vestibular neurons. Differential quantitative expression of *Kcnq4_v1* and *Kcnq4_v3* were respectively detected in the striolar and extra-striolar regions of the utricle and saccule. Analysis of gerbils and rats yielded results similar to those obtained in mice, suggesting that the spatiotemporal expression pattern of *Kcnq4* in the vestibular system is conserved among rodents. Analyses of vestibular HCs of *Bdnf* conditional mutant mice, which are devoid of any innervation, demonstrate that regulation of *Kcnq4* expression in vestibular HCs is independent of innervation.

Keywords

Vestibular hair cell; ion channel; Kcnq4; alternative splice variant; immunohistochemistry; immunofluorescence; in situ hybridization; RT-PCR

1. Introduction

The vestibular endorgans of the inner ear provide the dominant input for orientation in space to the brain. The gravistatic otolith organs, utricle and saccule, are the linear acceleration receptors, whereas the cristae of the three semicircular canals detect angular acceleration. Two different types of vestibular hair cells (HCs), type I and type II, can be distinguished in all five

*Corresponding author: Sonia M. Rocha-Sanchez, Ph.D., Creighton University School of Dentistry, Department of Oral Biology, 2500 California Plaza, Omaha, NE 68178, Tel: (402) 280-5098, Fax: (402) 280-5094, email: ssanchez@creighton.edu.

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vestibular endorgans. Three morphologically distinct types of afferent nerve fibers innervate the vestibular HCs: calyx afferents innervating type I HCs, bouton afferents innervating type II HCs, and dimorphic afferents innervating both HC types (Fritzsche, et al., 2001; Eatock, et al., 2002). The precise regional organization of the vestibular sensory epithelium and the progressive development of the HCs is well established (Watanuki and Schuknecht, 1976; Sans and Chat, 1982; Scarfone, et al., 1991; Goldberg, et al., 1992; Eatock, et al., 2002; Eatock and Hurley, 2003). In the mammalian otolith organs, the striolar region represents about 30% of the sensory epithelium and is predominately comprised of type I HCs with calyx afferent innervation; dimorphic innervated type I HCs and type II HCs are more abundant in the extra-striolar region. In rodents, the cristae can be subdivided into different zones (central, intermediate, and peripheral), with the central zone predominately containing calyx afferent innervated type I HCs (Scarfone, et al., 1991; Eatock and Hurley, 2003; Desai, et al., 2005a; 2005b).

Only limited information is available that describes the spatiotemporal expression patterns of genes present in the vestibular sensory epithelium (Chang et al. 2004; Fritzsche et al. 2006). However, similar to the cochlea, the developmental regulation of gene expression found in the vestibular epithelia appears to follow precise spatiotemporal patterns (Chang et al. 2004). Progressive upregulation and/or regional expression were documented for calcium-binding proteins, such as calretinin, calmodulin, and parvalbumin (Scarfone, et al., 1991; Rusch, et al., 1998; Desai, et al., 2005a; 2005b), neurofilament proteins (Desai, et al., 2005a; 2005b), ion channels (Fernandez, et al., 1988; Goldberg, et al., 1992; Beisel, et al., 2005) and *Kctd12*, a potassium channel tetramerization domain formally known as *Pfet1* (Resendes, et al., 2004). Cochlear expression of *Kcnq4*, a member of the *Kcnq* voltage-gated K⁺ ion channel gene family, in both neuronal and neurosensory cells has confirmed that *Kcnq4* expression is broader than initially proposed (Coucke, et al., 1999; Kubisch, et al., 1999; Kharkovets, et al., 2000; Beisel, et al., 2005; Hurley, et al., 2006; Kharkovets, et al., 2006). This essential requirement is now corroborated with the detection of *Kcnq4* transcripts and proteins in both inner and outer HCs as well as spiral ganglia neurons of mouse, rat, and gerbil cochleae (Kubisch, et al., 1999; Beisel, et al., 2000; Kharkovets, et al., 2000; Beisel, et al., 2005; Hurley, et al., 2006; Kharkovets, et al., 2006).

In the vestibular system, previous studies also indicated *Kcnq4* was highly expressed in vestibular HCs (Kharkovets et al. 2000). It was suggested that KCNQ4 detection only in type I but not in type II HCs was closely related to the presence of the calyx nerve ending innervating type I but not type II HCs. K⁺ channels, including, including KCNQ4, are critical elements in the development and functional maturation of cochlea and vestibular HCs (Valli, et al., 1990; Steinacker, et al., 1997; Marcotti and Kros, 1999; Si, et al., 2003). Most past evidences suggest that neither calyx formation nor innervation of any kind is required for electrophysiological and morphological differentiation and maturation of type I and type II HCs (Bianchi et al. 1996; Fritzsche et al. 1997; Rusch et al. 1998; Matei et al. 2005). Indeed, even the acquisition of *g_{K,L}* current in type I HCs, which is suggested to include KCNQ4 subunits, seems to be completely independent of innervation (Rusch et al. 1998). In contrast, recent evidence on KCNQ4 expression in vestibular epithelium implies a developmental expression regulation that appears to relate to afferent calyx innervation (Kharkovets et al., 1999; Hurley, et al., 2006).

Using whole mount immunohistochemistry (wmIHC) and immunofluorescence (wmIF), reverse transcriptase PCR (RT-PCR), and real-time quantitative PCR (QPCR), we provide, herein, a detailed description of the distribution and dynamic expression patterns of *Kcnq4* mRNA and protein in the vestibular sensory epithelia of mice at various stages of the development. Furthermore, we investigated whether or not the absence of afferent innervation and, consequently, of a physical calyx nerve ending would alter the expression of *Kcnq4*

subunits in type I HCs. WmIF analyses of KCNQ4 were performed using the floxed *Bdnf* conditional mutant mouse line, which lacks calyx nerve endings and nearly all vestibular neurons at postnatal stages (Bianchi, et al., 1996; Fritzsche, et al., 1997a; 1997b; Rios, et al., 2001). No differences were observed in the *Kcnq4* pattern of expression between mutant and wild type (WT) mice, which showed that type I and type II HCs staining is still comparable even in the complete absence of nerve fibers. These results resemble those previously described for KCNQ4 expression in the cochlear HCs (Beisel, et al., 2005) and demonstrate that in the vestibular HCs, as in the cochlea, *Kcnq4* is progressively upregulated during development.

2. Results

2.1. *Kcnq4* expression highlights the striola

KCNQ4 expression was barely detectable in the striolar region of the otolith organs in E18.5 mice (Fig. 1A). By P0 staining became more ubiquitous in the striolar HCs (Fig. 1B). During the postnatal maturation period KCNQ4 expression increased, such that by P21 expression appeared in HCs of the extrastriolar region as well (Fig. 1C, D). The pattern of immunoreactivity in the striolar region of utricles and saccules of mice P35 and older (up to P120; data not shown) was similar to P21. However, there was an increase in labeling intensity in the extra-striolar HCs in P35 and older mice compared to P21.

2.2. KCNQ4 is expressed throughout the cristae organs and vestibular neurons

A similar expression pattern was observed in the neurosensory epithelia of the three cristae of the semicircular canals. KCNQ4 expression was first detectable at P0 in the central zone HCs and extended to the entire sensory epithelium by P35 (Fig. 2A–D). These results demonstrate a differential regional expression pattern of KCNQ4, initially observed in the striolar and central zone type I HCs and, with age, progressing to all vestibular sensory HCs in the utricle, cristae and saccule (data not shown). A selective distribution of KCNQ4 and acetylated α -tubulin was observed in the vestibular ganglia of P21 mice (Fig. 3A–C), rat and gerbil (data not shown) and was similar to our previous results on the spiral ganglion neurons of the cochlea (Beisel et al., 2005). Further corroboration of these data came from the analysis of isolated vestibular HCs immunolabeled for KCNQ4 alone or double labeled for KCNQ4 and acetylated α -tubulin, which labels the nerve fibers entering the vestibular sensory epithelium. KCNQ4 positive signal was observed in both type I and type II HCs in the basolateral cell membrane as well as in the calyx nerve-ending of the type I HCs (Fig. 3D–F). The calyceal termination of the type I HCs showed a strong acetylated α -tubulin signal (Fig. 3F). WmIF analysis in P8 and P21 rat and gerbil vestibular endorgans (data not shown) showed an expression pattern similar to that in mice, suggesting that *Kcnq4* expression in the inner ear is conserved among rodents.

2.3. KCNQ4 immunodetection in BDNF conditional mutant (*lox/Cre*) mice

The ability of type I HCs to express *Kcnq4* even in the absence of a complete calyx nerve ending was tested in *Bdnf* conditional null mutant mice. These animals, early in their development, show absence of all afferent innervation to the anterior and posterior cristae and progressive loss of innervation to the otolith organs and horizontal crista (Bianchi, et al., 1996; Fritzsche, et al., 1997a; 1997b). WmIF was performed on the *Bdnf*^{2lox/2lox}/*Pax2-Cre* (*loxCre*) mice offspring at different time points (P8 and P14) and compared with aged-matched wild type (WT) animals using double-labeling with KCNQ4 and acetylated α -tubulin antibodies (Fig. 4A–F). Close examination of the neurofibers in *loxCre* mutant utricles showed reduction as well as disorganization of the neurofibers at P8 (Fig. 4D) and an almost complete absence of innervation at P14 (Fig. 4F). No differences were observed in the pattern of KCNQ4 immunoreactivity between WT and conditional mutant mice in the utricle (Fig 4A, C, and E).

Furthermore, KCNQ4 signal was detected in both WT (data not shown) and *loxCre* mutant horizontal and anterior semicircular canal cristae (Fig. 4G, H), which are completely devoid of afferent nerve fibers in the null mutant mice (Fritzsche et al. 1997). Overall the KCNQ4 expression pattern observed in the HCs of the vestibular endorgans of *loxCre* mice resembled that in the WT vestibular sensory epithelium, suggesting that similar to HC differentiation and maturation, KCNQ4 expression is independent of afferent innervation in all HC types.

2.4. Differential expression of *Kcnq4* splice variants

As shown in our previous study on cochlear *Kcnq4* expression, there is a differential utilization of the splice variants expression along the length of the cochlea (Beisel, et al., 2005). In order to identify the use of splice variants in the vestibular sensory epithelia, RT-PCR was used to quantitatively analyze the relative amounts of the four transcriptional splice variants of *Kcnq4* in the utricular macula (striola and extra-striola) and vestibular ganglion (Fig. 5A). *Kcnq4_v1* is the predominant form in the striolar and extra-striolar regions throughout development. However, a notable exception is observed at P21 in the extra striolar region, in which both *Kcnq4_v2* and *Kcnq4_v3* show relatively high expression levels. *Kcnq4_v3* predominates in the vestibular ganglion, especially in later maturational stages, while *Kcnq4_v4* is either at or below detection levels for all time points examined.

We looked for changes in the relative amounts of *Kcnq4* transcript in the striolar and extra striolar regions at three developmental times (Fig. 5B). In the striolar region, transcript levels increased from P0 to P8 and then sharply declined by P21. In the extra striolar region, transcript levels showed a steady increase from P0 through P21. Higher expression levels were generally observed in the extra-striolar region compared to the striolar region at the same developmental time points. This is opposite to the levels of protein, which appear to be higher in the striolar region throughout development (Fig. 1). The greatest differential expression levels between the striolar and extra striolar regions were found at P21. Comparison of our immunofluorescence and QPCR results suggest that differential turnover rates between *Kcnq4* transcripts and proteins occur over the entire vestibular neurosensory epithelia (compare Fig. 1 and 5A). Similar results have been also observed in the *Kcnq4* expression in cochlear sensory epithelia (Beisel, et al., 2005). An equivalent discordance between transcript and protein levels is found for *prestin* (Judice, et al., 2002; Zheng, et al., 2002) implying a slow turnover.

3. Discussion

3.1. HC specificity in the development regulation of *Kcnq4* expression

Previous work had suggested that type I HCs expressed KCNQ4 (Kharkovets, et al., 2000; Hurley, et al., 2006; Kharkovets, et al., 2006). We have confirmed and extended these studies and provided evidence that, in rodents, *Kcnq4* is topographically regulated in all HCs of vestibular sensory epithelia instead of restricted to a specific cell type. Strong KCNQ4 immunoreactivity was detected in the basolateral wall of both type I and type II HCs and afferent calyx endings innervating type I HCs. Significant levels of KCNQ4 immunoreactivity were also observed in the vestibular ganglia. These results are consistent with our previous KCNQ4 studies carried out in the murine cochlea (Beisel, et al., 2000; Beisel, et al., 2005), which showed *Kcnq4* varying expression levels in OHC, IHC, and spiral ganglion neurons. Except for the detection of KCNQ4 in the basal half of the type I HCs cell membrane and calyx nerve endings, labeling of the basolateral membranes of type I and type II HCs and vestibular ganglia neurons is documented here for the first time. Similar to our cochlea findings, we demonstrate that *Kcnq4* expression in the vestibular endorgans follows a developmental gradient with early upregulation in the striolar and central regions of the otolith organs and cristae of the semicircular canals, respectively. Although E16 mice were analyzed, *Kcnq4*

expression was only detectable at E18 in a few HCs in the striolar region of the utricular macula (Fig. 1A). In the extra-striolar and peripheral regions of the vestibular endorgans, *Kcnq4* expression was first detected at P8 progressing to higher levels during the maturational period. These results reinforce the idea that *Kcnq4* expression follows the time course for HC differentiation in the vestibular sensory epithelia (Ruben, 1967; Zheng and Gao, 1997; Fritzsche, et al., 2001; Eatock and Hurley, 2003). Likewise, several other genes have been described to be temporally (Zheng and Gao, 1997) and topologically (Shailam, et al., 1999; Goodyear and Richardson, 2002; Resendes, et al., 2004; Cristobal, et al., 2005) expressed in the vestibular neurosensory epithelia and neurons over time. Our findings demonstrate that *Kcnq4* has a spatiotemporal expression pattern in all vestibular neurosensory epithelia.

Regional qualitative and quantitative differences in *Kcnq4* protein and transcript levels are shown here in the developing vestibular endorgans and resemble the pattern described elsewhere for BK channels expression in the CNS (MacDonald, et al., 2006). *Kcnq4_v1* is the predominant form in the HCs of the organ of Corti throughout development. However, major variations are observed in the relative expression levels of the alternatively spliced forms at P21. As in the vestibular ganglia, *Kcnq4_v3* is the major form in the spiral ganglia, especially toward the basal region of the cochlea and is thought to be the *Kcnq4* variant associated with PHFHL (Beisel, et al., 2005). Overall, wIF and RT-PCR results in the vestibular system resemble that of the apical turn of the cochlea, which like the vestibular endorgans is a low frequency detector (Lewis, et al., 1985; Baird, et al., 1988; Beisel, et al., 2005; Highstein, et al., 2005). Divergence in splice form utilization between striolar and extra-striolar regions of the utricle at P8, which became more prominent at P21, could account for the differences in expression levels shown in the Fig. 5. *Kcnq4_v1* is the predominant form in the striolar region. Conversely, *Kcnq4_v2* and *Kcnq4_v3* seem to play a much larger role in the extra striolar region, particularly at later time points. These findings recapitulate *Kcnq4* expression in the organ of Corti, with large increases in expression of *Kcnq4_v1* during HC development and maturation and appearing to correspond with concomitant increases in the other splice forms. Alternative splicing is one mechanism utilized for generating a large number of mRNA and protein variants from a single gene and variation in the prevalence of these different forms may be indicative of their functional importance. We suggest that the conspicuous developmental changes in splice variant utilization, which we have observed in the vestibular system (this paper) and cochlea (Beisel, et al., 2005), strongly supports the hypothesis that *Kcnq4* channel splicing is coordinated in the developing ear and expression of distinct splice variants may allow cells to differentially modulate their electrical properties during development and in the adult HCs.

3.2. Type I and type II HCs morphophysiological maturation and innervation

In amniote vertebrates, type I and type II HCs can be differentiated by a number of features, including shape, size, innervation pattern and K^+ conductance (Lysakowski and Goldberg, 1997; Goldberg, 2000; Moravec and Peterson, 2004; Lopez, et al., 2005a; 2005b). However a number of evidences have suggested that the acquisition of some of these features, including morphological aspects, innervation pattern and even physiological characteristics of vestibular HCs may vary depending on the endorgan and their regional localization within the sensory epithelia rather than being specific to a HC type (Baird, et al., 1988; Highstein, et al., 2005; Lopez, et al., 2005b). Immunolocalization studies in the vestibular sensory epithelia suggest that *Kcnq4* expression was only detected in the membrane of type I HCs within the first postnatal weeks and more restricted to calyx inner face of those cells later on adulthood (Kharkovets, et al., 2000; 2006; Hurley et al. 2006). This proposal was substantiated by the detection of a negatively activating voltage-gated conductance, the $g_{K,L}$ current in the calyx endings of type I HCs (Kharkovets et al. 2000; Hurley et al. 2006) and reinforced by reports that both *Kcnq4* gene and $g_{K,L}$ current expression were not detected in embryonic or

neonatal cells but were acquired later in postnatal endorgans (Rusch, et al., 1998; Kharkovets, et al., 2000; Eatock, et al., 2002; Hurley, et al., 2006). The calyx nerve ending and $g_{K,L}$ current, the two distinguishing marks of type I HCs, resemble each other in their relatively late appearance in HC development (Linderman, 1973; Rusch et al. 1998; Wong et al. 2004) and their putative association to KCNQ4. However, we and others (Kharkovets, et al., 2000; 2006) have detected KCNQ4 in the vestibular HCs at much earlier time points than both $g_{K,L}$ acquisition and calyx formation, suggesting that its expression in the vestibular HCs is not associated to the presence of either one of the type I HCs hallmarks. Indeed, calyx formation and $g_{K,L}$ acquisition in type I HCs have been shown to be completely independent from each other (Steinacker, et al., 1997; Rusch et al. 1998). Furthermore, earlier reports on HC formation in mice lacking the neurotrophin receptors *Ntrk2* and *Ntrk3* have elegantly demonstrated that innervation is not required for HC formation and/or differentiation (Fritzsche, et al., 1997a; 1997b). More recently *Neurog1* null mice, which never develop sensory neurons, were shown to have full differentiated HC. Altogether these results reinforce the idea that innervation do not affect the ability of HCs to follow its own morphophysiological maturational course, however intact sensory HCs are necessary to maintain the cochlear and vestibular neurons (Fritzsche, et al., 1997a; 1997b). These results are in agreement with our present study where *loxCre* mouse vestibular HCs show a complete mature phenotype and still express KCNQ4 even in the absence of afferent innervation.

3.3. Vestibular *Kcnq4* expression and DFNA2

KCNQ4 potassium ion channels are known to play important physiological and pathological roles in the inner ear (Kunst, et al., 1998; Kubisch, et al., 1999; Beisel, et al., 2000; Beisel, et al., 2005). However, the molecular mechanism(s) behind the onset and development of the progressive deafness associated with mutations in this gene remains elusive. Dominant-negative mutations in KCNQ4 have been implicated in the pathophysiology of DFNA2 (Kunst, et al., 1998; Kubisch, et al., 1999). In general, *DFNA2* clinical features are consistent among patients: hearing impairment resultant from a *DFNA2* mutation is initially observed in the high frequency detection but becomes more accentuated and progresses to the low frequencies with age (Kunst, et al., 1998; Kubisch, et al., 1999; Beisel, et al., 2000; Beisel, et al., 2005). In contrast, *DFNA2* patients exhibit an apparently normal vestibular phenotype (Kunst, et al., 1998; Coucke, et al., 1999). It is likely that mutated KCNQ4 channels have altered electrophysiological function in vestibular neurons and HCs without overt vestibular dysfunction (Kharkovets, et al., 2006). Another possibility is that either other KCNQ family members may provide sufficient biological redundancy to retain normal vestibular function, as inferred from the analysis of genetically altered KCNQ4 mutant mice (Kharkovets, et al., 2000; 2006). Alternatively, similar to other types of progressive high frequency hearing loss (PHFHL), *Kcnq4*-mediated vestibular impairment in *DFNA2* patients might manifest itself as a function of age and/or rate of disease progression (De Leenheer, et al., 2002; Stern and Lalwani, 2002). More sophisticated dynamic tests of eye movement need to be performed on *DFNA2* patients to identify any KCNQ4 mutational effect in the canal cristae function. Understanding and clarifying the *Kcnq4* expression pattern in the ear is a very important step towards the complete understanding and eventual development of therapeutic approaches for the prevention and treatment of the *Kcnq4*-related deafness.

In summary, our data demonstrates that KCNQ4 is expressed in both vestibular neurons and neurosensory epithelia. KCNQ4 expression was observed in both types of vestibular HCs. However, *Kcnq4* expression was developmentally modulated with type I HCs of the striolar region showing earlier detectable KCNQ4 levels than type II HCs. These results were substantiated by immunofluorescence studies in *loxCre* mice, which show loss of all afferent innervation to the semicircular canals and progressive loss of innervation to the utricle and saccule. Based on our present study, we suggest that PHFHL pathology may also have a

vestibular component, which might become manifested in older patients. However, because of the broad expression of *Kcnq4* in the peripheral (Beisel, et al., 2000; Beisel, et al., 2005) and central auditory systems (Kharkovets, et al., 2000), we cannot rule out a role for the central auditory system in the onset and development of PHFHL.

4. Experimental procedures

4.1. Animal tissue and dissections

For the developmental analysis, inner ears from semi-outbred CF-1 mice were prepared as previously described (Beisel, et al., 2000; Beisel, et al., 2005). All animal tissue was harvested in accordance with the Creighton University Institutional Animal Care and Use Committee (IACUC) approved protocol number 0765 and 0630.1. *Kcnq4* expression was determined at every other embryonic (E) day from E16.5 up to neonates (P0) and at postnatal (P) days P15, P21, P35 and P120. At least 3 animals were analyzed for each time point. The endorgans were dissected without decalcification until P6. However, in order to facilitate the removal of the otoconia, the dissected otolith organs were rinsed in 0.1M EDTA/PBS, pH 7.4, for one hour. From P8 onward, the temporal bones were decalcified in the above mentioned solution for a variable period of time until sufficient decalcification was obtained. The creation of the *Bdnf*^{2lox/2lox} (*Bdnf*^{tm3Jae}) line is described by Gorski et al. (2003) and that of the *Pax2-Cre* (*Tg(Pax2-cre)IAkg*) transgenic line of Ohyama and Groves (2004). Mice with a targeted floxed *Bdnf* allele were bred with *Pax2-Cre* transgenic mice (Ohyama and Groves, 2004) to generate *Bdnf*^{2lox/+}/*Pax2-Cre* mice, which were then crossed to one another.

Subdissected tissue samples of the utricular macula were obtained by mechanical separation of the striolar and extra-striolar regions. Prior to each dissection, high power images of the utricular maculae were obtained by using a Leica DC 480 system attached to a Leica DC camera (Leica Microsystems Switzerland, Ltd). The striolar region was localized and boundaries of the striolar/extra-striolar regions were identified and used as markers for the dissections of these two regions. Due to the difficulty of precisely isolating these two regions, we do not discard the possibility of some extra-striolar contamination in the striolar sampling or vice versa; however we consider our parameters of dissection to be fairly consistent since different biological replicates gave similar results in QPCR. For single cell preparations, dissected pieces of utricular striolar and extra-striolar were treated in 1% papain for 5–10 minutes, briefly rinsed in 1X minimal essential medium (Gibco, Invitrogen Co.), transferred into uncoated gamma-radiated glass bottom petri dishes (MatTek Co., Ashland, MA), covered with a thin layer of 2.5% agarose and submitted to the immunofluorescence protocol described in section 4.3.

4.2. Total RNA isolation and PCR

Subdissected tissues were disrupted and homogenized using a PowerGen 35 homogenizer (Fisher Scientific, Pittsburgh, PA). Total RNA was isolated using the Qiagen RNeasy Mini Kit and then treated with DNase I (Ambion, Inc., Austin, TX). RNA purity was confirmed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Approximately 10 ng of each sample was used for QPCR, which was performed on the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). QPCR reactions were performed in triplicate using the Taqman One Step RT-PCR Kit (Applied Biosystems). Known quantities of mouse brain total RNA (Ambion) were used to establish a standard curve for relative quantification. The data were normalized utilizing an 18S ribosomal standard (Applied Biosystems).

In RT-PCR experiments, 100 ng total RNA was reverse transcribed using an oligo(dT)_{12–18} primer and Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). Amplification was performed using a PTC-200 Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA)

and FastStart Taq DNA Polymerase (Roche Applied Science, Indianapolis, IN). Reactions were 35 cycles using an annealing temperature of 55° C for 30 seconds and extension at 72° C for 2 minutes. Mouse *Kcnq4* splice variant-specific primers and QPCR primers used in these set of experiments were the same as described elsewhere (Beisel et al. 2005). The resulting PCR products were gel purified, blunt-ended using T4 polymerase, and ligated into the *HincII* site of a modified pBS^{+/−} phagemid vector (Stratagene, La Jolla, CA). The PCR-derived fragments as well as the subsequent cloned fragments were verified by direct sequence analyses using a CEQ 8000 Sequencer (Beckman Coulter, Fullerton, CA) and the thermosequencing ABI Prism Big Dye Terminator kit following the manufacturer's suggested protocol.

4.3. Immunohistological Analyses and Imaging

Immunofluorescence studies were done as described in Beisel et al. (2005) using rabbit antibodies against KCNQ4 N- and C- termini followed by a goat anti-rabbit secondary antibody conjugated to Alexa Fluor 568 (Molecular Probes, Eugene, OR). WmIHC and wmIF were performed in the vestibular endorgans of mice ranging from embryonic day (E) 16.5 to postnatal day (P) 120. However, due to low signal to background differences in the wmIF preparation of embryonic and early postnatal vestibular tissue, wmIHC was done instead using the Vectastain® ABC Kit (Vector Laboratories, Inc, Burlingame, CA). In addition, *loxCre* and WT control mice were submitted to double-labeling experiments with KCNQ4 antibodies, as described above and a monoclonal mouse anti-acetylated α -tubulin (Zymed Laboratories Inc.), followed by a rabbit anti-mouse secondary antibody conjugated to Alexa 647 (Molecular Probes, Eugene, OR). A confocal microscope (Zeiss LSM 510 Meta NLO, with LSM 5 image analysis software) was used for imaging of Alexa-conjugated secondary antibodies and morphometrical analysis.

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Abbreviations

HCS

	hair cells
IHC	inner hair cell
OHC	outer hair cell
wmIHC	whole mount immunohistochemistry
wmIF	whole mount immunofluorescence
E	embryonic day
P	postnatal day
QPCR	real-time quantitative PCR
PHFHL	progressive high frequency hearing loss

Figure 1.

KCNQ4 expression in the utricular macula of mice at different ages. At E18, KCNQ4 immunofluorescent signal was just above background levels (A). Close-up show some few KCNQ4 positive striolar HCs at E18. In P0 (B) and P8 (C) mice KCNQ4 upregulation started in the striolar region, and progressed toward the extra-striolar region as observed in P10 (D), P21 (E), and P35 utricles (F). White bar = 20 μ m.

Figure 2.

Developmental KCNQ4 expression in the vestibular cristae at different time points. Similar to the utricle, a progressive KCNQ4 expression was detected, starting at the central zone at P0 (A) and moving to the Peripheral Zone as shown in P8 (B), P21 (C), and P120 (D). Detail in B shows deep confocal scanning of the cristae sensory epithelia; asterisk indicates type I hair cells and their calyx ending both stained for KCNQ4. Because low level of detection was observed in wmIF of prenatal and P0 vestibular endorgans, enzymatic wmIHC was used to highlight KCNQ4 in the crista HCs. White signal = wmIF; Black signal = wmIHC; AC = Anterior Crista; PC = Posterior Crista. Bar = 20 μ m.

Figure 3. KCNQ4 expression analysis in CF-1 (P21) mouse vestibular ganglia neurons (**A–C**) and enzymatically isolated vestibular hair cells (**D–F**). Red (Alexa 568) = KCNQ4; Green (Alexa 647) = Tubulin; merging of the two colors is shown in **C**. **A–C**, bar = 20 μ m; **D–F**, bar = 10 μ m.

Figure 4.

These confocal images illustrate the differences and similarities between wild type (WT) and *Bdnf*^{2lox/2lox}/*Pax2-Cre* (*loxCre*) P8 and P14 mouse utricles. KCNQ4 (red) positive HCs and neurofibers (green) in the control P8 utricle (**A–B**). HCs were still present in the P8 utricle from *Bdnf* conditional mutant mice and expressed KCNQ4 (**C**); nevertheless, these animals exhibited much less and disorganized neurofibers (**D**) than the control animals. Similar results were observed in P14 *Bdnf* conditional mutant mice, in spite of the complete absences of afferent innervation (**E–F**). KCNQ4 positive HCs were also detected in the anterior cristae of P14 *Bdnf* conditional mutant mice, which is completely devoid of afferent fibers (**G–H**). Figures 4A–D are shown here at the same magnification. Bar = 20µm.

Figure 5.

Developmental expression of *Kcnq4* in the vestibular neurosensory and neuronal components. **A**, QPCR comparison of the striolar and extra striolar regions from postnatal P0, P8 and P21 mouse utricle. Contrasting with the immunofluorescence results, *Kcnq4* expression was higher in the extra-striolar region compared to the striolar, particularly at later time points. **B**, Developmental regulation of *Kcnq4* splice variants in the vestibular system is shown in subdivided utricle striolar (S), extra-striolar (ES), and vestibular ganglia (VG) of postnatal mice at different time points. A 1 kb ladder (Invitrogen, Carlsbad, CA) was used as a standard size marker (STD), and the corresponding kilobase lengths are indicated. Negative controls were a No Taq polymerase Control (NTC), a No Reverse transcriptase Control (NRT), and a No RNA template Control (NRC).