Chapter 9:

General Discussion

CHAPTER 9

GENERAL DISCUSSION

9.1 SUMMARY OF RESULTS

9.1.1 Mapping and sequencing progress

Through the discovery of new polymorphisms between the *bv* genetic background and the inbred strain 101/H which was used in the mapping backcross, the critical interval for *bronx waltzer* has been reduced by 0.09cM to 1.77cM and by 334Kb to 2.45Mb with the exclusion of seven candidate genes (Figure 3.30). This leaves 52 genes which have been mapped to the 2.6Mb region delineated by the flanking markers DASNP3 and *D5Mit209*, each of which are considered to be candidate genes for the *bronx waltzer* phenotype (Figure 3.32).

Following two rounds of primer design and sequencing, a total of 65729 base pairs of coding sequence, representing 90.63% of that lying within the *bv* candidate region have been successfully sequenced in the *bronx waltzer* mutant mouse genome and no mutations have been identified (Figure 7.7). Of the 52 genes mapped to the critical region full sequence has been obtained for 23 (Table 7.3), making these significantly less likely as candidates for the mutation.

9.1.2 Critical evaluation of candidate genes

The candidacy of each gene within the *bronx waltzer* region was assessed in Chapter 5, with consideration given to existing published literature and expression study data as well as to the results of an experiment to screen inner ear cDNA for the presence of candidate gene exons (Table 5.11). This led to the prioritisation of a subset of genes for further characterisation. The background literature and new data obtained for these genes is described in following paragraphs, and summarised below in Table 9.1.

Gene	Background Literature	Expression data	Zebrafish splice knockdown	Sequence coverage
Foxn4	Transcription factor controlling retinal development by activation of Math3, NeuroD1, and Prox1. Knockout has no hearing phenotype.	Expressed in retina and brain Not represented in expression databases Negative result in cDNA screen	Relatively normal development Positive tap test	100%
Myo1h	Myosin superfamily implicated in many inner ear abnormalities, including members of the myosin I class.	Not represented in expression databases Positive result in cDNA screen	Relatively normal development Positive tap test	97.2%
Kctd10	Voltage-gated potassium channel activity, potentially important in maintainiong ion gradients	Not represented in expression databases Positive result in cDNA screen	Development very stunted Fish not mobile	66.8%
Ube3b	Targets proteins for proteolytic degradation	Highly expressed following noise exposure Represented in 2 expression databases Positive result in cDNA screen	Variable rates of development Positive tap test	87.8%
Trpv4	Osmotically activated cation-selective channel. Drosophila orthologues involved in hearing. Knockout mice have progressive deafness.	Expressed in many tissues, including hair cells Represented in 1 expression database Positive result in cDNA screen	Normal development Positive tap test	100%
Git2	Regulation of golgi organisation, actin cytoskeletal organisation and paxillin localisation	Not represented in expression databases Positive result in cDNA screen	Variable rates of development Positive tap test	100%
Cabp1	Competitively inhibits calmodulin binding to CAMK2, possible role in Ca^{2+} regulation. May be involved in neuronal signal transduction	Represented in 2 expression databases Positive result in cDNA screen	Normal development Positive tap test	93.5%
Dnclc1	Inhibits the activity of neuronal NO synthase, implying regulatory role in numerous biologic processes	Represented in 3 expression databases Positive result in cDNA screen	Not tested	100%
Paxillin	Scaffolding protein involved in actin organisation Role in fibronectin and integrin signalling Knockout embryonic lethal, mesodermally derived structures affected	Represented in 3 expression databases Positive result in cDNA screen	Normal development Positive tap test	93.6%
Citron	Cit-K essential for cytokinesis in neuronal precursors Cit-N acts as Rho-modulator for actin-dependent organelle organisation	Represented in 2 expression databases Positive result in cDNA screen	Development stunted All fish dead by 3dpf	94.2%

Table 9.1: Summary of published and novel data regarding genes from within the *bronx waltzer* critical region identified as strong potential candidates

9.1.2.1 Forkhead box protein N4 (*Foxn4*)

Foxn4 is a member of the winged helix/forkhead family of transcription factors which is involved in many aspects of development (Gajiwala and Burley 2000). Expression of the gene has been noted during development in the retina and parts of the brain and spinal cord (Gouge *et al.* 2001) and subsequent studies showed that it is involved in the activation of *Math3*, *NeuroD1* and *Prox1* (Li *et al.* 2004).

Given the close evolutionary and developmental relationship between the ear and the eye as discussed in section 5.1.1.3, *Foxn4* presents itself as an interesting candidate. In addition, other winged helix/forkhead family members are known to be important in the development of the ear such as *Foxi1* (previously known as *Fkh10*). Mice with a targeted disruption of the *Foxi1* locus exhibit circling behaviour, poor swimming ability and abnormal reaching response as well as failing to elicit a Preyer reflex in response to a suprathreshold auditory stimulation (Hulander *et al.* 1998), a phenotype very similar to that displayed by the *bronx waltzer* mice.

The recent generation of *Foxn4* knockout mice by Li *et al.* (2004) showed that disruption of the gene affects retinal development but no hearing or balance defect was detected in the mice (M. Xiang, *pers. comm*). This makes it unlikely that *Foxn4* represents the causative agent for *bronx waltzer*, although these data were not available when candidates were selected for further investigation. In addition, during the course of this study, full sequence of *Foxn4* in *bv* mutant mice has been obtained and no were mutations identified.

9.1.2.2 Myosin 1H (*Myo1h*)

Given that some of the most crucial tissues of the inner ear are rich in actin and rely heavily for proper functioning on a correctly formed cytoskeleton (see Section 1.1.2.1), it comes as no surprise that several different myosin family members have been implicated in deafness since these molecular

motors are commonly known to interact with actin. Of the unconventional myosin isoforms, *myosin Ia, myosin IIIa, myosin VI, myosin VIIa* and *myosin XV* have all been shown to be associated with human genetic deafness loci (Libby and Steel 2000; Walsh *et al.* 2002). In addition, mutations in two members of the conventional nonmuscle myosin II family, *MYH9* and *MYH14* have also been demonstrated to give rise to hearing deficiencies, with *MYH9* being associated with Fechtner syndrome and DFNA17 (Seri *et al.* 2000) and *MYH14* being the gene responsible for the DFNA4 locus (Donaudy *et al.* 2004).

The myosin I class consists of the eight isozymes *Myo1a – Myo1h*, which have been implicated in various motile processes including organelle translocation, ion-channel gating and cytoskeleton reorganisation. All of these molecules were shown by Dumont *et al.* (2002) to be expressed in the mouse utricle by RT-PCR analysis with *Myo1b*, *Myo1c* and *Myo1e* exhibiting the highest expression levels. Myo1h was only sometimes amplified in the course of these experiments suggesting a lower and perhaps variable level of expression. However, this was also the case for *Myo1a* which has subsequently been linked with the DFNA48 locus (Donaudy *et al.* 2003), demonstrating that a lower expression level does not preclude an important role for *Myo1h* within the ear. *Myo1c* is considered to be the vertebrate orthologue of the bullfrog *myosin Iβ* and is thought to be involved in the gating of the mechanotransduction channel of sensory hair cells. Garcia *et al.* (1998) showed that *myosin Iβ* is localised near to the ends of the tip links in frog saccular cells as would be expected since these are the sites of adaptation. Further studies on *Myo1c* by Batters *et al.* (2004) demonstrated that the protein it encodes possesses a strain-sensing ADP release mechanism, allowing it to adapt to mechanical load and making it a good candidate for the mediator of slow adaptation of mechanoelectrical transduction.

Although relatively little is known about the functioning of *Myo1h* itself, its membership of the myosin superfamily which has been implicated in so many

and varied forms of inner ear abnormality makes it an interesting candidate gene. During the course of this work, 97.2% of the coding sequence has been sequenced in the *bronx waltzer* genome, with only one exon remaining to be sequenced and no mutations have been identified. The knockdown of the zebrafish ortholog using a splice blocking morpholino gave rise to larvae which developed normally and responded positively to the tap test, indicating that they had normal auditory function. These data make Myo1h less likely as a candidate for the *bv* mutation.

9.1.2.3 Potassium channel tetramerization domain-containing 10 (*Kctd10*)

Little is known about *Kctd10*, with no specific literature being currently available. However, its predicted potassium channel tetramerization protein domain and homology to other genes involved in potassium channel formation make it a potentially interesting candidate. The N-terminal, cytoplasmic tetramerization domain (T1) of voltage-gated K+ channels encodes molecular determinants for subfamily-specific assembly of alpha-subunits into functional tetrameric channels and is highly conserved in eukaryotes (Liu *et al.* 2005). As discussed in section 1.1.2.2, the maintenance of a potassium gradient is crucial for the proper functioning of the inner ear. In addition, other proteins associated with potassium transport have been shown to result in a deafness phenotype, including *KCNQ4*, a member of the voltage-gated potassium gene family which has been associated with the deafness locus DFNA2 (Kubisch *et al.* 1999). Meanwhile Jervell & Lange-Nielsen Syndrome has been demonstrated to be the result of mutations in the *KCNQ1* and *KCNE1* genes coding for the delayed rectifier potassium channel responsible for controlling endolymph homeostasis (Neyroud *et al.* 1997; Tyson *et al.* 1997) and mice with targeted disruptions in the *Kcnq1* gene displayed deafness and shaker-waltzer behaviour (Casimiro *et al.* 2001). Hence a novel protein with predicted involvement in potassium ion motility mechanisms presents as a good candidate for the *bronx waltzer* locus.

The sequence coverage achieved for this gene was 66.8%, and zebrafish larvae injected with a morpholino designed to block splicing in the ortholog showed very stunted development. These data are insufficient to rule out *Kctd10*, and thus it should still be considered a good candidate for *bronx waltzer*.

9.1.2.4 Ubiquitin protein ligase E3B (*Ube3b*)

Ube3b was initially identified in a screen examining differential gene expression following noise trauma in the chick basilar papilla. Lomax *et al.* (2000) found *Ube3b* to be highly expressed immediately after noise in the lesion, but not in the undamaged ends of the chick basilar papilla, suggesting that it may play a role in the regeneration of the specialised cells located there. Ubiquitination is a common process allowing molecules to be targeted for degradation by the 26S proteosome and requires the sequential action of three enzymes (Pickart 2000). An activating enzyme (E1), a conjugating enzyme (E2) and a ligase (E3) each act in turn to form polyubiquitin chains on the substrate protein. Vertebrates have a single E1 but may have several different E2s and E3s, allowing for a high degree of specificity in the targeting of proteins for degradation (Kumar *et al.* 1997). Lomax *et al.* (2000) found UBE3B to be expressed strongly in the pituitary gland and the heart, and at lower levels in all tissues examined, indicating that it may play a role under normal conditions as well as under stress and making it unlikely that its functionality should be confined to the ear. Gong *et al.* (2003) noted that the *C.elegans* homologue *Oxi-1* was identified in a screen for oxidative stress (Yanase and Ishi 1999), suggesting that *UBE3B* may play a role in the stress response triggered by oxidative damage following noise trauma. These findings, whilst not pointing to an obvious developmental function for *Ube3b* in the ear, do suggest that it plays a role in the ear and make it a possible candidate for *bronx waltzer*.

The sequence coverage achieved for this gene was 87.8%, and zebrafish larvae injected with a morpholino designed to block splicing in the ortholog showed

variable rates of development but reacted positively to the tap test, suggesting normal auditory function. These data make *Ube3b* less likely as a candidate for *bronx waltzer*, but do not rule it out.

9.1.2.5 Transient receptor potential cation channel V4 (*Trpv4*)

The transient potential cation channel superfamily V is made up of non-specific cation ion channel receptors gated by heat, protons, low extracellular osmolarity and arachidonic acid derivatives. *Trpv4* has been shown to be osmotically activated, described by Liedtke *et al.* (2000) as a cation-selective channel gated by exposure to hypotonicity. The maintenance of osmotic gradients is crucial for the proper functioning of the inner ear as discussed in Section 1.1.2.2, making this molecule an interesting candidate for the *bv* mutation, especially since Liedtke *et al.* reported expression of *Trpv4* in the hair cells of the inner ear amongst other tissues. Furthermore, the related ion channel Nanchung (*Nan*) in Drosophila was demonstrated to be necessary for hearing by Kim *et al.* (2003). They found that *Nan* mediates hypo-osmotically activated calcium influx and cation currents in cultured cells. In vivo it was shown to be expressed exclusively in chordotonal neurons and localised to the sensory cilia. Mutants lacking *Nan* showed absent sound-evoked potentials, suggesting a key role for the protein in the chordotonal mechanotransducer.

They hypothesised that *Nan* may be the Drosophila orthologue for *Trpv4* based on the expression of both molecules in mechanosensory cells and their activation by cellular hypotonic stress. By sequence homology though, it was found to be equally similar to *TRPV1*, suggesting that it belongs to the same family but does not necessarily represent a direct orthologue. Indeed, the generation of *Trpv4* knockout mice by Mizuno *et al.* (2003) suggests that *Trpv4* plays a role distinct from that of the proposed Drosophila orthologue. Mice lacking *Trpv4* were observed to have impaired osmotic sensation (Mizuno *et al.* 2003), impaired pressure sensation (Suzuki *et al.* 2003) and altered thermal

selection behaviour (Lee *et al.* 2005). They were later observed to develop a hearing deficit, with mice at 8 weeks having normal hearing but those at 24 weeks displaying significantly higher thresholds by auditory brainstem response (ABR). They were also found to be more susceptible to acoustic injury than wild type controls, showing a significantly larger auditory threshold shift one week after exposure (Tabuchi *et al.* 2005). The cause of this hearing loss is as yet unknown, and it would be interesting to examine the sensory epithlia of *Trpv4* knockout mice in order to establish this. These data have only recently come to light, and although they confirm that *Trpv4* plays a role in inner ear function and thus was a good candidate for *bronx waltzer,* the described phenotype differs significantly from that of *bv* mutant mice. In *bronx waltzer* mice the inner hair cells fail to mature at all, suggesting a disruption in their development rather than in their maintenance as is suggested by a phenotype of late-onset deafness and vulnerability to acoustic injury. In addition, the *Trpv4* knockout mice do not display any of the behaviour typical of those with vestibular abnormality (see Section 3.1.1.4) which might be expected if the gene were the same in both cases.

Zebrafish larvae injected with a morpholino designed to block splicing in the ortholog showed variable rates of development but reacted positively to the tap test, suggesting normal auditory function. In addition, the full coding sequence of *Trpv4* has been examined in the *bronx waltzer* mutant genome and no mutations have been identified, making *Trpv4* a much less attractive candidate as the causative agent for *bv*.

9.1.2.6 G protein-coupled receptor kinase- interactor 2 (*Git2*)

Git2 is an ADP-ribosylation factor GTPase-activating protein (ARFGAP) which has been shown to interact with *paxillin* (Mazaki *et al.* 2001), a protein important in actin organisation and intracellular signalling (see section 9.2.1.9). The authors found that overexpression of the short isoform of *Git2* caused the redistribution of Golgi protein β-COP and reduced the numbers of

paxillin-containing focal adhesions and actin stress fibres. In particular, the role of *Git2* in the regulation of actin cytoskeletal organisation could indicate a function for it in relation to the stereocilia on the apical surfaces of sensory hair cells within the inner ear whose structure depends on the arrangement of actin filaments and without which hearing and vestibular function are greatly compromised. In addition, Premont *et al.* (2000) found that the human homologue *GIT2* undergoes extensive alternative splicing and exists in at least 10 and potentially as many as 33 distinct forms, thus opening up the possibility that a mutation in a particular exon may cause a very specific phenotype, despite expression being detected in all tissues examined. During the course of this study the full annotated sequence of *Git2* has been analysed in *bv* mutant DNA and no mutations identified. Knockdown of the zebrafish ortholog gave rise to larvae which developed abnormally but which responded positively to the tap test, suggesting normal auditory function. It is therefore unlikely that *Git2* represents the causative agent for *bronx waltzer*, but since this gene shows a great deal of variability in its manifestation in different tissues, it is plausible that another exon as yet unidentified is necessary for the proper functioning of *Git2* in the ear, and therefore it should not be fully ruled out.

9.1.2.7 Calcium-binding protein 1 (*Cabp1*)

CABP1 was first identified as a human brain cDNA clone showing similarity to the calmodulin family of calcium binding proteins (Yamaguchi *et al.* 1999) and was later demonstrated by Haeseleer *et al.* (2000) to be capable of substituting functionally for calmodulin (*CaM*), a protein whose functions include roles in growth and the cell cycle as well as in signal transduction and the synthesis and release of neurotransmitters. This suggested a role for *CABP1* in the central nervous system where it may augment or substitute for CaM. Subsequently, Lee *et al.* (2002) used co-immunoprecipitation assays to demonstrate modulation of a rat P/Q-type voltage-dependent Ca^{2+} channel by *CABP1* through interaction with the alpha-1 subunit. They postulate that

this interaction between Ca^{2+} channels and *CaBP1* may regulate Ca^{2+}-dependent forms of synaptic plasticity by inhibiting Ca^{2+} influx into neurons. In order for hair cells in the inner ear to function correctly they are surrounded by fluid containing calcium at concentrations that must be maintained by active transport, hence a protein involved in the regulation of calcium channels may potentially have a role in the ear. Some examples which demonstrate the importance of controlling calcium in the inner ear include the deaf, ataxic mouse deafwaddler mice with mutations in the *Atp2b2* gene encoding plasma membrane calcium ATPase type 2 (*PMCA2*) which have been shown to result in reduced Ca^{2+} concentration in the endolymph (Wood *et al.* 2004), and mice deficient for the alpha1D Ca^{2+} channel which have impaired hearing but no vestibular defect (Dou *et al.* 2004).

Cabp1 is thought to be neuron specific and is expressed strongly in the retina (Sokal *et al.* 2000). As previously discussed (see Section 5.1.1.3) expression of a gene in the eye can sometimes indicate a role for it in the ear, and the expression of *Cabp1* in the ear has not been investigated. This suggestion, along with a potential role in Ca^{2+} regulation makes this a possible candidate gene. During the course of this work, 93.5% of the coding sequence has been sequenced in the *bronx waltzer* genome, with only one exon remaining to be sequenced and no mutations have been identified. The knockdown of the zebrafish ortholog using a splice blocking morpholino gave rise to larvae which developed normally and responded positively to the tap test, indicating that they had normal auditory function. These data make *Cabp1* less likely as a candidate for the *bv* mutation.

9.1.2.8 Dynein light chain 1, cytoplasmic (*Dnclc1*)

Cytoplasmic dyneins are large, multi-subunit ATPases involved in the transport of particles and organelles along microtubules and in the transport of condensed chromosomes during mitosis. These molecular motors function by interacting with microtubules to generate force and are encoded by a

number of separate genes. Of these, the best characterised are the cytoplasmic heavy chains which share extensive sequence similarity and are conserved throughout species. More recently, several genes encoding intermediate and light chains have been identified and have been found to encode a remarkable diversity of products, which also seem to be highly conserved between species, although they fall into several complex groups (Milisav 1998). Light chains are thought to play roles in cargo-binding as well as in regulatory control of the dynein motor (King 2000).

DLC1, the human form of *Dnclc1*, was shown by Dick *et al.* (1996) to be ubiquitously expressed and localised to the cytoplasm. The authors also demonstrated that mutations in the Drosophila orthologue ddlc1 resulted in pleiotropic morphogenetic defects in bristle and wing development, as well as in oogenesis. Meanwhile, Jaffrey and Snyder (1996) found that human *DLC1* inhibits the activity of neuronal nitric oxide, a major messenger molecule in the cardiovascular, immune, and nervous systems, suggesting that *DLC1* may play a regulatory role in numerous biological processes.

The full sequence of *Dnclc1* has been obtained during the course of this study and no mutations identified in the sequence from *bv* mutants, making it unlikely that this gene represents the causative agent for *bronx waltzer*.

9.1.2.9 Paxillin (*Pxn*)

Paxillin localises to focal adhesions (FAs), specialised sites where cells adhere to the extracellular matrix (ECM) (Turner *et al.* 1990). This process is mediated by integrin receptors which connect intracellular actin to the ECM, and it is thought that FAs are required for the maintenance of actin organisation within the cell (Jockusch *et al.* 1995; Burridge and Chrzanowska-Wodnicka 1996) as well as mediating tyrosine kinase signalling relating to growth control (Aplin *et al.* 1998). These data suggest roles for FAs in the regulation of cell morphology, migratory properties, growth and

differentiation (Burridge and Chrzanowska-Wodnicka 1996). In keeping with this broad functionality, *paxillin* knockout mice were embryonic lethal with defects as early as E7.5 and resorbed by E9-9.5. (Hagel *et al.* 2002). *Paxillin* was found to be involved in the development of mesodermally derived structures such as heart and somites, while cultured *paxillin*$^{-/-}$ fibroblasts displayed abnormal FAs, reduced cell migration and a reduction in the levels of other FA-related proteins, as well as showing defects in the cortical cytoskeleton and cell spreading on fibronectin. Since the mice do not develop beyond E9.5, no studies of the inner ear have been possible.

With respect to the *bronx waltzer* phenotype, the role of paxillin in actin organisation marks it out as a potentially interesting candidate gene. The stereocilia of the sensory hair cells which are affected by the *bv* mutation are packed with actin filaments which maintain their structure and are constantly renewed by means of a molecular treadmill (Rzadzinska *et al.* 2004). The shape of these structures is crucial to their operation (see Section 1.1.2.1), and thus any disruption to the organisation of actin in the cell may well cause the cell to cease functioning, leading to downstream apoptotic events. Apoptosis may also result from a loss of paxillin-mediated signalling, or by the loss of cell adhesion normally provided by FAs.

Paxillin is a multi-domain protein which has been shown to bind to with several structural and signalling proteins within FAs (Turner and Miller 1994; Hildebrand *et al.* 1995; Brown *et al.* 1996). This property may provide one possible explanation for how a mutation in such a ubiquitously important gene could give a phenotype as specific as that seen in *bronx waltzer* mice. A mutation in a single binding domain could potentially cause the protein to malfunction only when it is required to interact with a specific molecule, or even a particular combination of molecules whose purpose once conjugated is specific to inner hair cells. Alternatively, the tissue specificity may result from an alternative splice form or promoter site which results in a slightly different configuration of the protein, as discussed in Section 9.1.3.

During the course of this study, 93.6% coverage of the coding sequence for *paxillin* has been analysed in the *bronx waltzer* background and no mutations identified, with only one exon remaining to be sequenced. The knockdown of the zebrafish ortholog using a splice blocking morpholino gave rise to larvae which developed normally and responded positively to the tap test, indicating that they had normal auditory function. These data make *paxillin* less likely as a candidate for the *bv* mutation, but do not rule it out.

9.1.2.10 Citron (*Cit*)

The *Citron* transcription unit gives rise to two isoforms – Citron-K and Citron-N – produced via alternative transcriptional initiation (Di Cunto *et al.* 2000). Citron-K is the longer of the two variants since it comprises an additional amino-terminal serine/threonine kinase domain (Di Cunto *et al.* 1998). Citron-K shares a high degree of homology with the Rho-kinases (ROCKs) (Leung *et al.* 1996) with its activity stimulated by activated Rho-A (Di Cunto *et al.* 1998) and evidence suggests it is involved in the control of cytokinesis downstream of Rho (Madaule *et al.* 1998). Citron-K knockout mice were found to suffer developmental abnormalities in the central nervous system as a result of altered cytokinesis and massive apoptosis (Di Cunto *et al.* 2000). Although the ears of these mice have not been fully examined, they show no behaviour indicative of deafness or vestibular abnormality and the vestibulo-cochlear receptors of these mice appeared superficially normal (Di Cunto, *pers. comm.*). In addition, other mutations generated in the same gene gave rise to very similar phenotypes, making it unlikely that a mutation in the Citron-K transcription unit would result in isolated deafness (Di Cunto, *pers. comm.*).

The functions of Citron-N are less well characterised, although it was first identified for its ability to interact with the GTP-bound (active) forms of Rho and Rac (Madaule *et al.* 1995; Fujisawa *et al.* 1998) and has been proposed as a link between the Rho signalling cascades and NMDA receptor complexes

(Zhang *et al.* 1999). Rho is an important molecule in focal adhesions (see section 9.1.2.9), being the trigger for their formation and for the assembly of actin filaments (Ridley and Hall 1992), while Rac has been shown to be involved in the polymerisation of actin filaments in lamellipodia (Nobes and Hall 1995; Nobes and Hall 1995). Camera *et al.* (2003) carried out analysis of hippocampal neurons in culture which indicated that Citron-N controls actin in the Golgi apparatus by assembling the Rho effector ROCK-II and the actin-binding, neuron-specific protein Profilin-IIa. They postulated that Citron-N acts as an organelle-specific Rho-modulator for actin-dependent organelle organisation and dynamics. This is an interesting proposition, since as has been previously discussed the proper organisation of actin filaments is vital to the functioning of inner ear hair cells. While the current literature suggests that Citron-N is brain specific, expression in the ear has not been explicitly examined. In addition the fact that *Citron* demonstrates alternate mechanisms of transcription raises the possibility that another isoform may exist which could be specific to the hair cells of the inner ear, and is required for actin organisation within them. Some methods for testing this hypothesis are outlined in Section 9.2.3.

During the course of this study, 94.2% of the coding sequence of *Citron* has been analysed in the *bronx waltzer* mutant genome and no mutations have been found. Three exons from a total of 51 remain to be sequenced, with two of these lying within the region of the gene which is common to Citron-N and Citron-K. Zebrafish larvae injected with a morpholino designed to block splicing in the ortholog showed very stunted development. These data are insufficient to rule out *Citron*, and thus it should still be considered a good candidate for *bronx waltzer*.

9.1.3 Tissue specific expression

Many of the candidate genes examined in the paragraphs above, whilst having functions suggestive of a role in the ear also show widespread expression or involvement in many other important pathways. This seems to be in conflict with the very restricted nature of the *bronx waltzer* phenotype, which affects only the sensory hair cells of the inner ear. Even more specifically, in the cochlea only the inner hair cells are affected with the outer hair cells appearing normal, suggesting a very precise role for the mutated gene. In these cases, the possibility of tissue-specific modification of gene expression must be considered. Most commonly, distinct isoforms of a gene are produced by alternative splicing in individual cells or tissues, with a varying combination of exons being retained in the processed mRNA and thus a different protein produced following translation (reviewed in Smith *et al.* 1989). Another possible mechanism is that of alternative transcriptional regulation. Here, cell-specific expression is achieved by the activation of different control elements such as promoters or termination sequences, resulting in a pre-mRNA of differing composition. For example, the activation of different promoters associated with the α and γ clusters of neural protocadherin (*Pcdh*) genes causes the incorporation of a variable first exon which is then spliced to the remainder of the gene following transcription (Wu and Maniatis 2000; Tasic *et al.* 2002). A detailed study of the mouse *UGT1* cluster showed that its genomic organisation is very similar to that of the *Pcdh* clusters, and that the various isoforms are expressed in a tissue-specific manner (Zhang *et al.* 2004). Analysis of mammalian Genbank databases (Zhang *et al.* 2004) and *Arabidopsis* full-length gene transcripts (Haas *et al.* 2002) has indicated that multiple transcription initiation sites are widespread within eukaryotic genomes and are much more common than was previously thought, making this an important mechanism in regard to cell- and tissue-specific, as well as developmentally regulated gene expression.

In addition to providing a possible explanation for the specificity of the *bronx waltzer* phenotype, this mechanism also suggests a potential reason for the failure to detect a mutation within the coding regions of the candidate genes despite achieving over 90% coverage in the mutant genome. If the mutation were to lie within a promoter or other regulatory element then exon-resequencing would not reveal it. An example of such a mutation is the dinucleotide deletion in the promoter region of the gene *ankyrin* which causes hereditary spherocytosis as a result of abnormal gene expression (Gallagher *et al.* 2005).

Furthermore, although the splice junctions at either end of each exon were examined during this study, mutations within an intron may occasionally cause a phenotype if they disrupt the normal function of transcription or of mRNA processing. For example, a cryptic splice donor site may be activated, as in the case of mutations in intron 2 of *CDKN2A* which have been associated with six English melanoma pedigrees (Harland *et al.* 2001). Alternatively, the splice machinery may be disrupted by a mutation in a lariat branchpoint consensus sequence, such as the point mutation located in intron 4 of *lecithin:cholesterol acyltransferase* (*LCAT*) which gives rise to fish-eye disease (Kuivenhoven *et al.* 1996), or by the expansion of an unstable polymorphic repeat such as that found in intron 1 of the gene FRDA which results in the autosomal recessive disease Friedreich's Ataxia (Campuzano *et al.* 1996).

The expression studies described in Section 9.2.3 should allow these possibilities to be fully investigated.

9.2 FURTHER WORK

Through limitations imposed by time, not all of the avenues opened up by the present work have been fully explored. Suggestions for extensions to the described work, as well as for novel areas of investigation are outlined in the paragraphs below.

9.2.1 Further mapping

Sequence traces obtained from the resequencing of exons within the *bronx waltzer* candidate region (see Chapter 7) brought to light new data regarding polymorphisms which could potentially be exploited for mapping purposes. The majority of these were SNPs or length polymorphisms between the *bv* genetic background and the publicly sequenced strain C57Bl/6J. In order to establish whether these could be used to aid in the mapping of *bronx waltzer*, they should be sequenced in the backcross strain 101/H and that sequence compared to the *bv* sequence. Where polymorphisms between these two strains exist, they can then be tested against the panel of recombinant mice and placed as new markers on the *bv*/101 genetic map (see Chapter 3).

The second type of polymorphisms identified during exon resequencing were those where SNPs existed between the *bronx waltzer* mutant (*bv*/*bv*) sequence and the wild type (+/+) sequence derived from the *bronx waltzer* genetic background. In each case the allele from the mutant sample matched that of the published sequence, suggesting that the base substitutions were not pathogenic. Interestingly, all the SNPs of this type were clustered around the gene *Citron*, indicating a possible linkage disequilibrium effect. In order to test this hypothesis, archived samples from the *bronx waltzer* colony could be tested for the markers to establish whether they do indeed segregate with the mutation. If so then further sequencing and characterisation efforts should be concentrated on this region.

9.2.2 Further sequencing

Coding sequence coverage of 90.63% in the mutant genome has been achieved, and yet no mutation has been identified. It is possible that the mutation lies within the remaining 9.37% of the annotated coding sequence. It may also be the case that it lies within a coding region which has not yet been annotated, or even in a non-coding region as discussed in Section 9.1.3.

The quality of published sequence and gene annotation in the *bronx waltzer* region is currently not high. Few of the selected tiling path clones have had their sequence fully finished, and there exist two gaps in the tiling path which are bridged by lower quality whole genome shotgun data. Proper annotation of coding regions is dependent on a high quality of sequence, and thus the finishing of the tiling path clones is imperative for a confident analysis and characterisation of the candidate region. Negotiations are underway to ensure the prioritisation of this work.

Following two rounds of sequencing, 90.63% of the annotated coding regions of genes within the *bv* candidate region have been analysed in the mutant genome and no mutations have been identified. Of the 52 genes annotated, full sequence has been obtained from the mutant genome for 23, with the majority of the remaining 29 having partial sequence available. Many of these genes lack sequence for only one or two exons, making the prospects for a third sequencing pass very favourable. The successful sequencing of a relatively small number of exons in the mutant genome could allow the coding sequence of a significant number of genes to be completed, thus reducing the list of those to be considered as likely candidates for the mutation. Detailed suggestions for the implementation of this third round of sequencing can be found in Section 7.4.1.

9.2.3 Further expression studies

In assessing candidacy, the expression data considered for each gene was obtained from a variety of sources – published literature, cDNA databases and the screening of cDNA obtained from P0 ears using gene-specific primers. This last data source was a relatively simple experiment, with much scope for refinement. Since the gene-specific primers have already been obtained and optimised, the process of screening a new, more specific cDNA sample would be relatively simple. Suggestions for the procurement of RNA

which better represents the mRNA population of the ear and has a lower level of contamination from other tissues are detailed in Section 5.4.1.2.

An alternative approach to this study which would provide more detailed data regarding the level and location of expression would be to carry out high throughput *in situ* hybridisation. Probes designed to hybridise to the mRNA product of each candidate gene would be incubated with inner ear tissue from a developmental stage at which the bronx waltzer gene is expected to be expressed, such as at E17.5 – E19.5 when the hair cells are maturing and when the phenotype becomes apparent. In order to facilitate the screening of a large number of genes and also to provide more detailed information regarding the cell types in which a gene is expressed, it would be advantageous to carry out hybridisation to sections of ear tissue rather than to whole ears (Wilkinson 1992).

In order to investigate the possibility of the mutation lying in a non-coding regulatory element it would be useful to carry out a number of Northern blot experiments. The first of the proteins expressed in the inner ear versus other tissues in order to establish whether an ear-specific isoform exists, and the second of mutant versus control samples to identify whether the transcription or splicing of a gene differs between the two. In both cases probes should be designed to a region of the gene likely to be included in all isoforms, such as one containing a domain necessary for its functioning, while at the same time being specific to the gene rather than a gene family. If any anomalies were identified, these could be investigated using 5' and 3' RACE (Rapid Amplification of cDNA Ends) to recover any differences in the incorporation of exons at the beginning or the end of a gene. Localised sequencing of the non-coding regions could then be carried out in order to identify the mutation.

9.2.4 Gene knockdowns and knockouts

Although none of the gene knockdowns using morpholinos in zebrafish (see Chapter 6) gave rise to larvae exhibiting a hearing or balance phenotype, the success of the negative and positive controls indicates that this is a strand of investigation worth continuing. Firstly, it is important that the efficacy of the morpholinos is confirmed using the RT-PCR experiment described in Section 6.4 to ascertain what effect the splice MOs have had on their corresponding gene products. Once this technique has been validated, the potential for the zebrafish to be used as a second confirmatory allele could be quickly and easily exploited in the event of the identification of a mutation in the *bv* genome. The additional advantage of a zebrafish phenocopy allowing a fuller investigation into the developmental effects of the mutation as a result of the transparent and externally maturing larvae lends further support to the pursuance of this approach.

A second means of investigating gene function by targeted disruption is the generation of mouse knockouts. This can be a relatively slow process since it requires the breeding of several generations of mice before the phenotype can be assessed, however the current establishment of a large scale programme at the Sanger Institute to generate and characterise around 250 such mouse mutants per year could provide the opportunity to focus on this approach. These will be generated using a combination of gene-trapping and homologous recombination recombineering techniques, both of which support a forward genetics approach i.e. the identity of the disrupted gene is known before mice are produced. Gene-traps function by randomly targeting genes in embryonic stem (ES) cell lines (Skarnes *et al.* 2004), while recombineering techniques allow the targeted inactivation of genes which are not amenable to trapping in ES cells (Liu *et al.* 2003). Depending on available capacity, a larger or smaller subset of the candidate genes for *bronx waltzer* could be prioritised in this study to allow investigation of the effects of their disruption. In addition, this could prove a highly useful resource when the *bv*

mutation is identified, as a means of proving that it is indeed the causative agent for the phenotype.

9.3 *BRONX WALTZER* AS A MODEL OF HEREDITARY DEAFNESS

As well as holding insights into the development and function of the inner ear sensory epithelia, and particularly into the molecular differences between inner and outer hair cells, *bronx waltzer* mice serve as a potential model for human hereditary deafness. The human homologs of the genes from within the *bv* candidate region on mouse chromosome 5 are localised to a region of human chromosome 12 which spans from 12q24.11 to 12q24.31. This region forms part of the mapped interval for the deafness locus DFNA25 which has been localised to 12q22-24 (Greene *et al.* 2001; Petek *et al.* 2003). It has been hypothesised that this form of human congenital deafness is the human ortholog of *bronx waltzer* (Greene *et al.* 2001), and some of the same candidate genes have been examined. One of these was *VR-OAC*, now renamed *TRPV4*, which as discussed in Section 9.1.2.5 has given rise to a progressive deafness phenotype when knocked out in the mouse, although the cell types affected by the deficiency have not been established. Since DFNA25 also manifests as delayed-onset sensorineural hearing loss, it seems that *TRPV4* is a good candidate for this dominant hereditary hearing impairment locus. However, it is difficult to reconcile these clinical and laboratory observations with the *bronx waltzer* phenotype, where inner hair cells and vestibular hair cells fail to develop to maturity, and affected mice are deaf from birth. Congenital neuroepithelial progressive hearing impairment tends to be the result of mutations in genes required for the maintenance and function of the sensory patches, rather than for their proper development (reviewed in Gratton and Vazquez 2003). In addition, when degeneration of the organ of Corti is observed, the order of hair cell loss is generally from base to apex, with OHCs degenerating first, later followed by IHCs. This pattern is markedly different from that reported in *bronx waltzer* mutants, where only

IHCs are seen to degenerate and OHCs appear normal even as the mice grow old. Furthermore, during the course of this study the full coding sequence of *Trpv4* has been examined in the *bv* genome and no mutations identified. Thus it is likely that *bronx waltzer* still represents a novel locus which when mutated causes disruption to the normal development of the auditory system, and holds the potential for new discoveries concerning the development of the ear.