Chapter 8:

Modifiers of the

bronx waltzer *mutation*

CHAPTER 8

MODIFIERS OF THE *BRONX WALTZER* MUTATION

8.1 INTRODUCTION

8.1.1 Variability in the *bronx waltzer* phenotype

Although the phenotype of *bronx waltzer* mice has been well described (see Section 1.4), studies over the years have pointed to a level of variability in the severity of the mutation when manifested on different genetic backgrounds. In particular, the number of surviving inner hair cells in the organ of Corti in *bv/bv* mutants has been observed to range from as low as 5% (Keithley and Feldman 1983) to as high as 30% (Schrott *et al.* 1989). In addition, variability has been observed in the occurrence and frequency of atypical hair cells, these being hair cells with the appearance of outer hair cells but which are located in the inner hair cell row.

During the initial mapping of *bv*, a backcross to *Mus castaneus* gave rise to *bv/bv* homozygotes with variable and often significantly reduced behavioural phenotypes (Bussoli and Steel, unpublished observations). While this rendered the backcross inappropriate for genetic mapping purposes owing to the difficulty in determining the genotype of the mice at the *bv* locus by behavioural observation, it suggests that the *Mus castaneus* genome may contain one or more dominant modifiers of the *bronx waltzer* phenotype. This is an interesting proposition, since the identity of the *bv* gene is unknown and thus so are the pathways within which it operates. The identification of molecules which exert a modifying effect on the manifestation of the *bronx waltzer* phenotype may well provide clues to the function of the gene itself.

8.1.2 A backcross to *Mus castaneus*

In a previous study, Nogueira (2000) generated a backcross [(*bv*/*bv* x *Mus castaneus*) F1 x *bv*/*bv*] of 241 mice and performed behavioural tests to assess the severity of the phenotype in the mice as compared to a control panel consisting of 14 F1 (+/*bv*) mice, 15 *bronx waltzer* (*bv*/*bv*) mice and 14 *Mus castaneus* (+/+) mice. The tests were carried out on mice aged six to seven weeks and were based on the standard battery developed by Hardisty *et al.* (1997) to provide a quantitative measure of vestibular abnormalities. They included behavioural observation in an open field arena, contact righting response, reaching response and a test for swimming ability, with each mouse being assigned a score of 1 for behaviour similar to that expected from wild type mice and a score of 0 if their behaviour matched that of *bv*/*bv* mice. In addition the Preyer reflex was tested using a click-box as an assessment of hearing ability, with a strong reflex being scored as 2, a weak one as 1 and an absent one as 0. As an example of the variability in phenotype, Figure 8.1 shows some of the results from the open field test. The number of squares traversed is used as a measure of the hyperactive behaviour which is often exhibited by mice with vestibular dysfunction. Wild type mice tend to investigate cautiously, traversing a relatively small number of the marked squares and keeping to the outside of the space. This can be seen in Figure 8.1 where very few of the F1, *Mus castaneus* or heterozygote mice from the backcross traversed more than 299 squares. The heterozygous backcross mice showed a very restricted distribution to between 0 and 199 squares, while the homozygous mutant backcross mice showed a broad variation. Some of them traversed more than 1000 squares whilst others entered fewer than 100, overlapping with the number of squares traversed by heterozygous mice. The swimming, contact righting and reaching response tests each showed clear evidence of variation within the group of homozygous backcross mutants (data not shown).

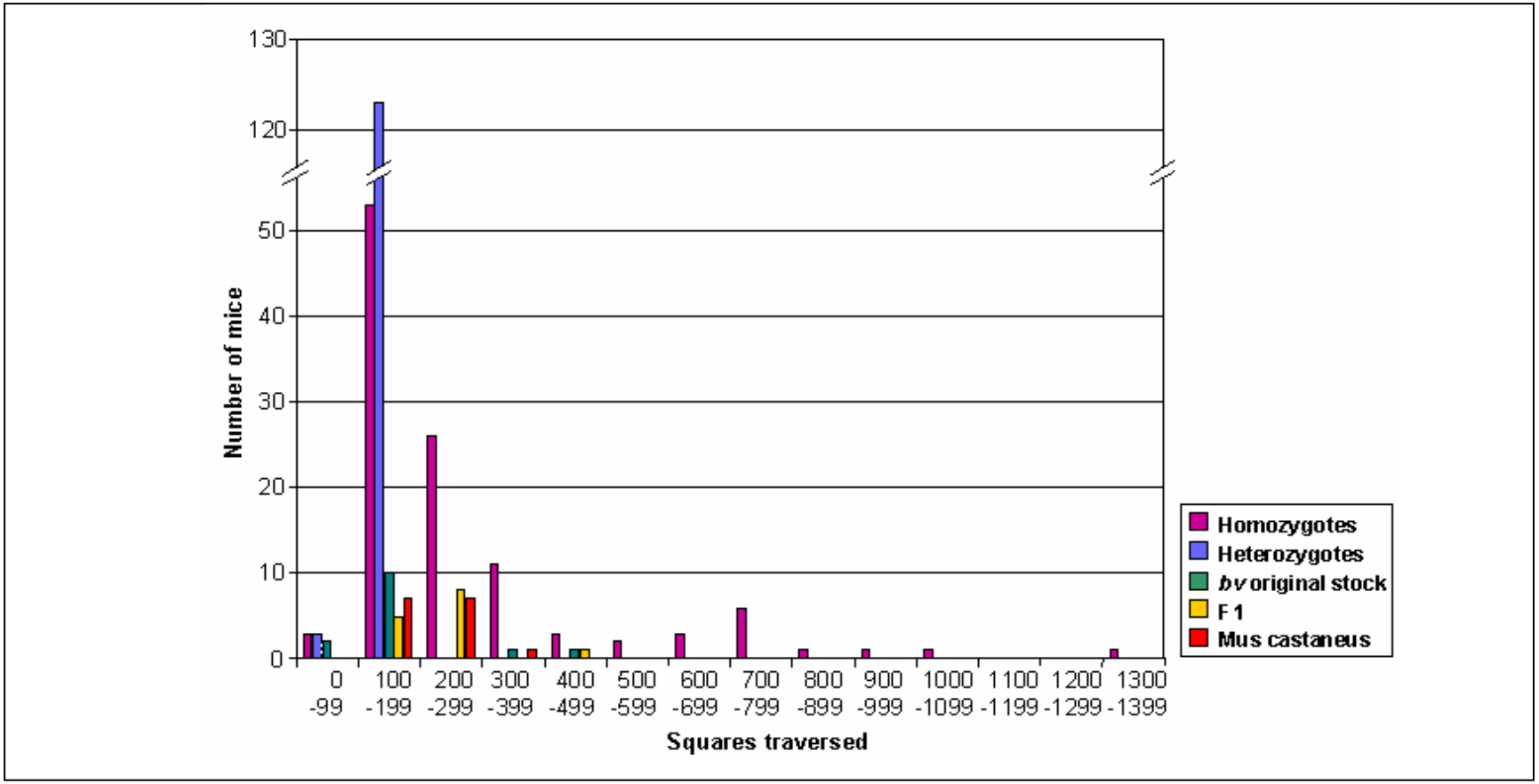

Figure 8.1: Bar chart illustrating the number of squares traversed by mice originating from the *bronx waltzer*/*Mus castaneus* backcross as compared to control mice in an open field arena. Homozygous mutants from the *bv*/*cast* backcross show a very wide distribution for this trait, several behaving similarly to control mice while others traverse many more squares than mice from the original *bv* stock. Data from Nogueira (2000).

8.1.3 Linkage analysis of behavioural trait modifiers

The behavioural testing was carried out blind to genotype, with typing later carried out on DNA samples from each mouse using the flanking markers from the *bv*/101 backcross, *D5Mit25* and *D5Mit209*. Linkage analysis was initially performed on the 30 homozygous backcross mice with the highest and lowest total scores in the hearing and balance tests. This approach was adopted because selection of individuals with extreme phenotypes as opposed to genotyping all the individuals significantly increases power over random sampling to detect linkage with modifiers (Lander and Botstein 1989; Darvasi *et al.* 1993; Darvasi and Soller 1994; Darvasi 1997). A genome scan using 76 simple sequence repeat length polymorphic markers was carried out on these 30 mice in order to determine linkage to modifiers, and the markers *D4Mit27*, *D5Mit101*, *D5Mit408*, *D12Mit274* and *D12Mit64* exhibited a significant, low P-value (below 0.05) while *D4Mit125*, *D9Mit224* and *D17Mit49* exhibited a slightly higher P-value (between 0.05 and 0.1) using the χ^2 test.

In the second stage of modifier analysis, markers exhibiting nominal significance ($p<0.1$) in the initial genome scan (chromosomes 4, 5, 9, 12 and 17), together with further polymorphic markers in these regions, were typed in the total set of 113 homozygous backcross *bv*/*bv* mice. Mapmaker QTL (Lander *et al.* 1987) analysis for each of the traits was carried out at this stage rather than the χ^2 test. In this analysis of linkage, a probability (P) $\leq 1.0 \times 10^{-4}$ (LOD 3.3) was considered as significant linkage and a P $\leq 3.4 \times 10^{-3}$ (LOD 1.9) as suggestive linkage (Lander and Schork 1994; Lander and Kruglyak 1995). Of the nine traits analyzed over the five chromosomes, ten combinations showed suggestive or significant linkage indicating the possible location of modifier loci. LOD score plots representing these loci are presented in Figure 8.2. Only LOD scores at a distance of 0cM from each marker used are plotted, because Mapmaker QTL otherwise maximizes predicted LOD scores between markers. The percentage of the variance of

the trait explained by the genotype at each marker is given at the top of each plot.

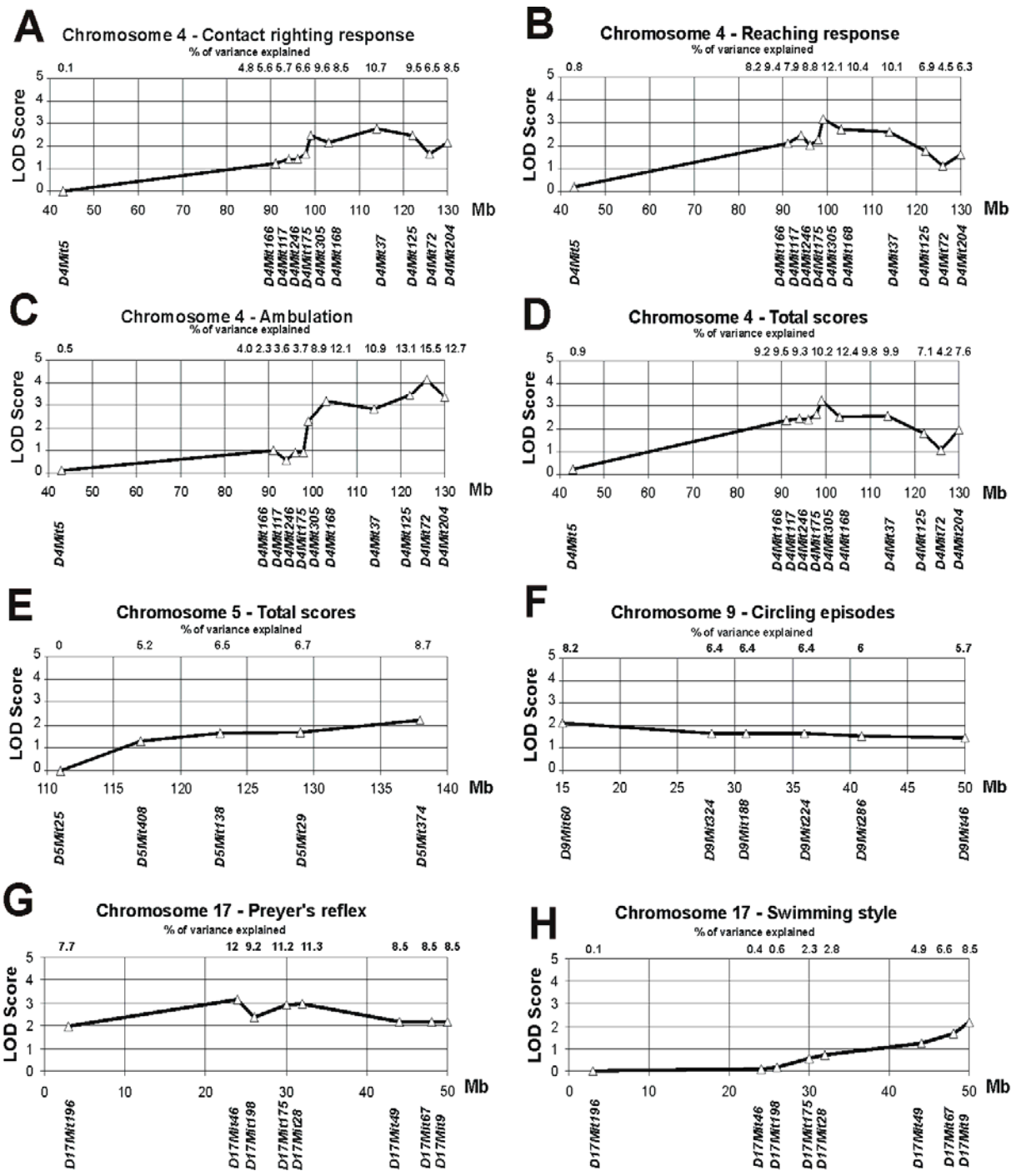

Figure 8.2: LOD score plots showing the possible locations of modifier loci identified in a backcross of *bronx waltzer* to *Mus castaneus* (Nogueira 2000). A significant modifier was found to map to chromosome 4 near *D4Mit72* affecting ambulation in the open field tests (C). The remaining graphs show loci which were found to be suggestive modifiers (LOD score >1.9) for the traits tested.

Five traits showed linkage to chromosome 4. A broad region associated with ambulation was detected with the peak near *D4Mit72* (LOD 4.14, accounting for 15.1% of the variance in phenotype; Fig. 8.2C). The large interval with raised LOD scores may suggest the presence of more than one modifier in distal chromosome 4. A suggestive modifier (LOD 3.26 accounting for 12.4% of the variance) associated with variation in total scores of all traits mapped to central chromosome 4, near *D4Mit305* (Fig. 8.2D). Two other traits showed a suggestive linkage to the same region near *D4Mit305*: the reaching response (LOD 3.16 accounting for 12.1% of the variance; Fig. 8.2B) and total balance scores (LOD 3.18 accounting for 12.1% of the variance; data not shown). The fifth trait mapping to chromosome 4 was the contact righting response, with a peak at *D4Mit37* (LOD 2.77 explaining 10.7% of the variance; Fig. 2A). On chromosome 5, *D5Mit374* showed suggestive linkage with a peak LOD score of 2.2 explaining 8.7% of the variance for the total scores as well as for the total balance scores (Fig. 8.2E). On chromosome 9, only circling episodes showed linkage, with a suggestive modifier near *D9Mit60* (LOD=2.1 explaining 8.2% of the variance; Fig. 2F). No traits showed any evidence of linkage to chromosome 12, with the highest LOD score reaching only 0.61. Finally, on chromosome 17, two traits showed suggestive linkage: the Preyer reflex, with a broad region with LOD scores above 2 and a peak at *D17Mit46/D17Mit80* (LOD 3.13 explaining 12.0% of the variance; Fig. 8.2G), and swimming style with a peak LOD score of 2.19 at *D17Mit9* explaining 8.5% of the variance (Fig. 2H). The only remaining trait, swimming vigour, showed no evidence of modifier loci.

8.1.4 Hair cells in backcross mutants

The analysis so far carried out on the mice arising from the *bronx waltzer/Mus castaneus* backcross has been entirely behavioural in nature, and whilst this is often a good indicator of vestibular abnormality and the standard battery of tests allow it to be somewhat quantifiable, there remain further measures of the severity of the *bv* phenotype which have yet to be explored. The most

obvious of these is the number of surviving inner hair cells within the organ of Corti, since this has previously been observed to vary when the mutation is hosted on different genetic backgrounds, and since the loss of inner hair cells is one of the most defining features of *bronx waltzer* mutant mice. In addition, the data obtained up to this point are mainly concerned with the measurement of vestibular function, with only the Preyer reflex being used as a measure of hearing ability. This test is relatively crude and will only detect a profound hearing impairment, since a mouse with a mild hearing deficit is still able to respond to sound.

Thus, in the present study, the survival of IHCs and the frequency of atypical hair cells in a sample of *bv*/*bv* mutants from the *bv*/cast cross at the extremes of observed behaviour (normal and severely affected) will be examined using scanning electron microscopy of the organ of Corti. These will be used as further measures of the penetrance of the phenotype on the *Mus castaneus* background, and the data subjected to QTL analysis in order to determine whether linkage can be described between these measures and any of the existing or any new modifier loci.

8.2 METHODS

8.2.1 Phenotypic extremes

This analysis was carried out on the 30 homozygous backcross mice with the highest and lowest total scores in the hearing and balance tests, referred to as the mice at the phenotypic extremes. The balance tests were carried out by assigning homozygous mice that performed in a similar way to heterozygous mice a score of 1, and homozygous mice that displayed a behavior outside the normal range of the heterozygous mice a score of 0. Thus high scoring mice are those which behave most like wild type mice, while low scoring mice exhibited the behaviour most typical of mutant mice. The traits analyzed to detect any modifier effect were contact righting response, swimming vigour, swimming style, reaching response, ambulation and number of circling episodes (Noguiera, 2000). In addition, the Preyer reflex was analyzed using an extended scoring system, giving mice with a good reflex a score of 2, mice with a reduced reflex a score of 1, and mice with no response a score of 0.

8.2.2 Cochlear dissection

At the time the backcross animals were sacrificed in order to obtain material for the genome scan, the inner ears of each mouse were removed from the skull and fixed as described in section 4.2.4. They were then stored for a period of years in cocodylate buffer (0.1M sodium cacodylate; 2mM $CaCl_2$) at 4^{o}C and during this time some of the samples became fragile or were colonised with fungal matter, making them unsuitable for further analysis. For the remaining samples which were in good condition, dissection of the outer cochlear shell and tectorial membrane was performed in order to allow the apical surface of the organ of Corti to be viewed using scanning electron microscopy.

8.2.3 Preparation of samples for scanning electron microscopy

Fixed cochleas were processed for SEM using the osmium tetroxide-thiocarbohydrazide (OTOTO) method (Hunter-Duvar 1978; Self *et al.* 1998). They were first washed in 1% osmium tetroxide in cocodylate buffer (0.1M sodium cacodylate; 2mM $CaCl_2$) for one hour before being washed in ddH_2O 6 times for 5 minutes each. There followed a wash in freshly dissolved, saturated thiocarbahydrazide in water for 20 minutes, followed by a further 6 washes in ddH_2O for 5 minutes each. A wash in 1% osmium tetroxide in water for 1 hour was followed by another series of 6 washes in ddH_2O for 5 minutes, and this was followed by a second 20 minute wash in thocarbohydrazide, with 6 washes in ddH_2O for 5 minutes. Finally a third wash for one hour in osmium tetroxide in water was performed, with the excess rinsed away in a series of 6 washes in ddH_2O for 5 minutes each.

Having been fixed and coated, the samples were next dehydrated in a dilution series of acetone washes, each of 15 minutes. Washes were performed at 70%, 80%, 90% and three washes were carried out in 100% acetone. Once fully dehydrated, the samples were dried in a critical point drier (Polaron) and were mounted on aluminium SEM stubs (TAAB) using silver conductive paint (Agar Scientific, Stansted, Essex). These were then coated with a fine layer of gold using a sputter coater (Polaron) prior to being visualised using a Phillips XL30 scanning electron microscope. Specimens were routinely examined at a working distance of 10mm, at an accelerating voltage of 10kV, and with a spot size of 3nm.

8.2.4 Hair cell counts

SEM images were obtained from between 30-50% (mid-basal turn) and 70-90% (apical turn) of the total length of the cochlear duct from the base to the apex. These regions were determined by measuring and dividing the visible portion of the cochlea into nine equal parts corresponding to between 10% and 100% of the length, with the first 10% being made up by the hook which is not visible when the cochlea is viewed from above. These images were then used to construct montages covering approximately 300μm of the length of the organ of Corti, and from these the number of inner hair cells and atypical hair cells per 100μm was calculated.

8.3 RESULTS

8.3.1 Hair cells in backcross mutants

Control mice showed a full complement of IHCs (14-15 IHCs/100μm) and no sign of atypical hair cells (data not shown). Amongst the mutants, there was a large variation observed in the numbers of remaining IHCs and the numbers of atypical hair cells (hair cells within the inner hair cell row but with a hair bundle like that of outer hair cells) as shown in Figure 8.3. Inner hair cells and atypical hair cells were counted at two locations along the length of the cochlear duct, mid-basal (30-50% of distance from base) and apical (70-90% of distance from base) turns, the distributions of counts are shown in Figure 8.4. Mutants with the lowest balance scores showed fewer IHCs and more atypical hair cells than mutants with the highest balance scores (Table 8.1). The balance score was calculated by the summation of all the phenotypic test scores except for the Preyer reflex (see Section 8.2.1).

Cells per 100μm	Lowest balance scores	Highest balance scores	p-value
IHCs in base	1.373 +/- 0.4577 (Range 0.26 – 3.76) (n=10)	11.344 +/- 2.0617 (Range 3.44 – 15.26) (n=7)	0.0029
IHCs in apex	1.93 +/- 0.3315 (Range 0.89 – 3.22) (n=8)	11.951 +/- 1.9185 (Range 5.55 – 15.01) (n=6)	0.001
Atypical hair cells in base	4.253 +/- 0.806 (Range 1.79 – 9.00) (n=10)	0.396 +/- 0.287 (Range 0 – 1.06) (n=7)	0.0006
Atypical hair cells in apex	1.651 +/- 0.488 (Range 0 – 3.74) (n=8)	0.106 +/- 0.0747 (Range 0 – 0.37) (n=6)	0.0081

Table 8.1: Comparison of hair cell counts between mutants from the two phenotypic extremes.

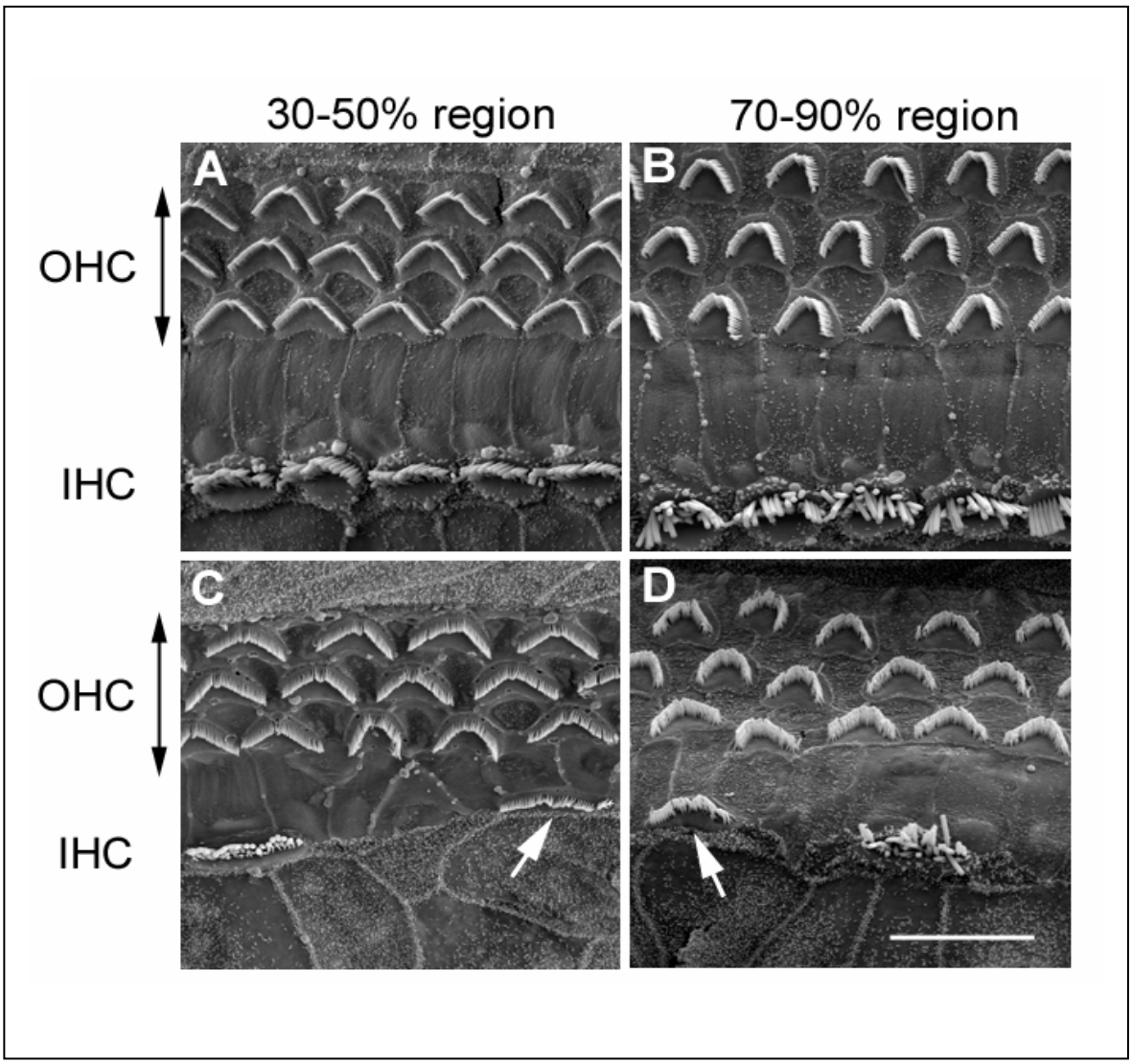

Figure 8.3: Scanning electron micrographs showing apical surfaces of cells in the organ of Corti reveal variation between mutants at the extremes of observed phenotype.
A,B high scoring mutants, C,D low scoring mutants.
Most homozygous mutants which scored highly in the behavioral tests showed an arrangement of hair cells similar to that seen in control mice. Both in the mid-basal 30-50% region (A) and the apical 70-90% region (B) there are three rows of outer hair cells (OHC) and a single row of inner hair cells (IHC). In mutants having low scores in the behavioral tests many IHCs are missing, causing some disruption of the surrounding support cells and OHCs. Atypical hair cells are also observed (indicated by arrows) in the mid-basal 30-50% region (C) and the apical 70-90% region (D) of low scoring mutants. These are cells which have the appearance of OHCs but are present in the row where IHCs are expected to be situated.
Scale bar = 10μm

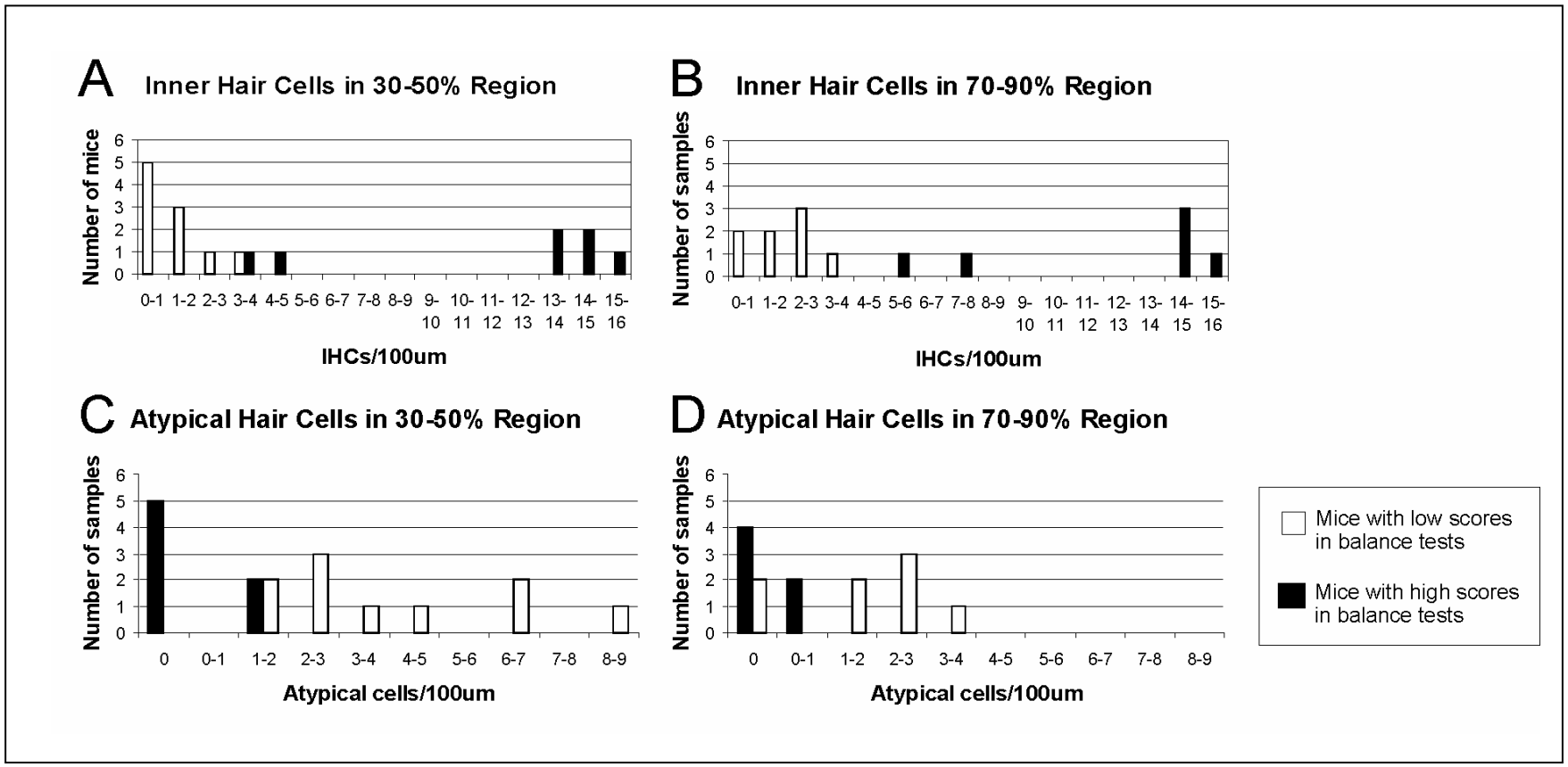

Figure 8.4: Distributions of inner hair cell and atypical hair cell counts
Numbers of inner hair cells and atypical hair cells in the cochleae of mice from the phenotypic extremes. The two groups show clear segregation both in relation to the number of inner hair cells (A and B) and the number of atypical hair cells (C and D) present.

Statistical analysis was carried out using t-tests to compare counts for both cell types at the two cochlear locations investigated revealed significant differences between the two phenotypic extremes (see Table 8.1). Furthermore, among the ten mutants examined by scanning electron microscopy with the lowest balance scores, 5 had a Preyer reflex and 5 showed no reflex, and there was a significant difference in IHCs numbers between these two groups, with those with no Preyer reflex showing fewer IHCs (see Table 8.2).

	With Preyer reflex	**No Preyer reflex**	**p-value**
IHCs in base	2.078 +/- 0.472 (n=5)	0.669 +/- 0.188 (n=5)	0.025
IHCs in apex	2.456 +/- 0.286 (n=5)	1.070 +/- 0.146 (n=3)	0.0043
Atypical hair cells in base	2.889 +/- 0.552 (n=5)	5.616 +/- 1.182 (n=5)	0.075
Atypical hair cells in apex	1.130 +/- 0.518 (n=5)	2.519 +/- 0.670 (n=3)	0.19

Table 8.2: Comparison of hair cell counts between mutants with and without a Preyer reflex among the group with the lowest balance scores

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8.3.2 Linkage analysis of hair cell modifiers

The IHC and atypical hair cell counts at the two cochlear locations were used as four further traits to study by χ^2 analysis and by MapmakerQTL, to detect linkage of modifiers of these traits. For atypical hair cells, mice with none were given a score of 1 and mice with some a score of 0 in the analysis. For IHC counts, the actual numbers of IHCs/100 μm were used for the χ^2 analysis, and for MapmakerQTL analysis an additional score of 1 for 3.0 or more IHCs/100 μm and 0 for fewer than 3.0 IHCs/100 μm was assigned. By χ^2 analysis, markers on chromosomes 4, 5, 9, 10, 11, 12, 15, 17 and 19 all showed p values below 0.05 for at least one of the four traits (Table 8.3). IHCs in the base showed the largest number of potential modifier loci. By MapmakerQTL analysis, only one of these loci reached the criterion for suggestive linkage to a modifier, *D15Mit63* for IHCs in the base, with a LOD score of 2.05 explaining 42.6% of the variance. Several other markers reached LOD scores over 1.2 (Table 8.4), corresponding broadly to the χ^2 probabilities. The relatively small numbers of samples available probably contributed to the lack of significant LOD scores.

Marker	IHCs base	Atypicals base	IHCs apex	Atypicals apex
D4Mit27	0.0295			
D5Mit80	0.0269	0.0031		
D5Mit155	0.0153			0.0241
D5Mit101	0.0429			
D9Mit60		0.0358		
D9Mit46	0.0269			
D10Mit43				0.0308
D10Mit237		0.0493		0.0157
D11Mit358				0.0308
D12Mit64	0.0153			
D12Mit274	0.0269			
D15Mit49	0.0295			
D15Mit63	0.0071			
D17Mit9	0.0295	0.0358	0.0308	

Table 8.3: P values from χ^2 analysis of markers associated with hair cell traits ($p<0.05$ only included)

Trait	LOD	Variance explained	Marker
Preyer reflex	1.887	40%	*D4Mit305*
IHCs in base	1.326	30.20%	*D4Mit305/168/37*
IHCs in base	1.254	28.80%	*D9Mit46*
Atypicals in apex	1.233	33.40%	*D10Mit43*
Preyer reflex	1.255	28.80%	*D15Mit63*
IHCs in base	2.050*	42.60%	*D15Mit63*
IHCs in base	1.568	34.60%	*D19Mit45/48*
Atypicals in base	1.359	30.80%	*D19Mit45/48*

Table 8.4: Peak LOD scores for Preyer reflex and hair cell traits among the mutants from the phenotypic extremes (LOD >1.2 only included). An asterisk indicates that the locus meets the criterion for suggestive linkage to a modifier

8.4 DISCUSSION

This study identified a large amount of variation in the hair cell counts from mutants of the two phenotypic extreme group. As the phenotypic extreme groups were determined on the basis of their balance scores reflecting vestibular function and hair cell counts reflected the effects upon the cochlea, the difference between the phenotypic extremes in hair cell counts suggested that the two systems (auditory and vestibular) are subject to some extent to similar influences of modifiers (Table 8.1). As both systems depend upon inner ear hair cell function, this is not too surprising. The observation of more IHCs and fewer atypical hair cells in mice with a Preyer reflex compared with those without amongst the group of mutants with the lowest balance scores (Table 8.2) suggests that the Preyer reflex can reflect hair cell status in this situation, although the number of mice studied is low. It was interesting that the chi-squared analysis and LOD scores using hair cell counts as traits indicated some new potential modifier loci (albeit at low likelihoods) that were not detected by the behavioral trait analysis (Tables 8.3 and 8.4). In particular, the only marker that reached the criterion for suggestive linkage with a LOD score of 2.05 (for IHCs in the base) was on chromosome 15 at *D15Mit63*, a chromosome that was not picked out by the behavioral test results. A number of these potential modifier loci may be spurious results, resulting from the relatively low numbers of samples available to study hair cells, so these putative loci should be regarded with some caution.

In the initial study, all of the putative modifier loci demonstrated a broad distribution of LOD scores along the respective chromosomes (Figure 8.2). This is probably due to the relatively small number of mice used (113) leading to limited opportunity for informative recombinations to accrue and define the locus more precisely. Furthermore, each modifier is likely to exert an effect that is less than fully penetrant upon the phenotype, and this will interact with the variance inevitably associated with any behavioral measurements to reduce the precision of the mapping. It is also possible that

there is more than one putative modifier located in each chromosomal region, and several of the LOD score plots show secondary peaks.

The number of putative modifiers detected in this work is very likely to be an underestimate of the total number, for several reasons. Only can detect modifiers that happen to segregate in the two parental strains used will be detected, and two modifiers that are closely linked will be difficult to segregate as mentioned above. Modifiers may have positive or negative effects upon the phenotype, and may show different patterns of inheritance and only dominant modifiers can be detected using the existing backcross. This may complicate interpretation and reduces the chances of detecting some modifiers. Finally, there may be many loci each with a small effect upon the phenotype, which will make detection of any of them difficult. The calculations of relatively small proportions of the phenotypic variance explained by the putative modifiers in this study (e.g. 15.5% of the variance for our most significant locus, *D4Mit72*) supports a model including several modifiers with additive effects. However, it is not uncommon to find values of less than 10% of the variance explained by individual modifiers, and a value of 20% may be considered a locus of major effect (Tanksley 1993).

Further work on the putative modifier loci will be needed to verify the loci and ultimately to identify the genomic polymorphisms underlying the modifier effects. Several approaches can be used, including refining the mapping of the loci by increasing the number of mice studied, developing congenic lines to further define the modifier locations, and using transgenic rescue techniques or large scale sequencing when candidate regions are narrowed down to a handful of genes. These approaches have been successful in identifying other modifiers (e.g. Johnson *et al.* 2001; Ikeda *et al.* 2002). However, these will all be difficult approaches to adopt when it is clear that there is not a single modifier of major effect in the case of the *bronx waltzer* phenotype. It may be sensible to adopt another approach in this case: to await reports of good candidates from the large-scale mouse mutagenesis

programmes and microarray expression studies that are identifying many new genes involved in hearing and deafness, and to specifically test these new candidate genes by typing intragenic polymorphisms provided by SNP consortia efforts.