Heterozygosity Mapping of Partially Congenic Lines: Mapping of a Semidominant Neurological Mutation, wheels ( whl), on Mouse Chromosome 4

Patrick M. Nolan
Indiana University of Pennsylvania

Patricia J. Sollars
University of Nebraska-Lincoln, patricia.sollars@unl.edu

Barbara A. Bohne
Washington University School of Medicine

Warren J. Ewens
University of Pennsylvania

Gary E. Pickard
University of Nebraska-Lincoln, gpickard2@unl.edu

Follow this and additional works at: http://digitalcommons.unl.edu/vetscipapers

Part of the Biochemistry, Biophysics, and Structural Biology Commons, Cell and Developmental Biology Commons, Immunology and Infectious Disease Commons, Medical Sciences Commons, Veterinary Microbiology and Immunobiology Commons, and the Veterinary Pathology and Pathobiology Commons

http://digitalcommons.unl.edu/vetscipapers/248

This Article is brought to you for free and open access by the Veterinary and Biomedical Sciences, Department of at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Papers in Veterinary and Biomedical Science by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.
Authors
Patrick M. Nolan, Patricia J. Sollars, Barbara A. Bohne, Warren J. Ewens, Gary E. Pickard, and Maja Bucan
Heterozygosity Mapping of Partially Congenic Lines: Mapping of a Semidominant Neurological Mutation, Wheels (Whl), on Mouse Chromosome 4

Patrick M. Nolan,* Patricia J. Sollars,* † Barbara A. Bohne, ‡ Warren J. Ewens,§ Gary E. Pickard*** †† and Maja Bućan* †††

Departments of *Psychiatry, §Biology, **Neuroscience, ††Genetics, and †††Center for Sleep and Respiratory Neurobiology, University of Pennsylvania, Philadelphia, Pennsylvania 19104 and ‡Department of Otolaryngology—Head and Neck Surgery, Washington University School of Medicine, St. Louis, Missouri 63110

Manuscript received October 11, 1994
Accepted for publication January 26, 1995

ABSTRACT

We identified a semidominant, chemically induced, mouse mutation with a complex array of abnormal behaviors including bidirectional circling and hyperactivity, abnormal circadian rhythmicity and abnormal responses to light. In this report, we genetically and phenotypically characterized the circling/waltzing component of the abnormal behavior. We mapped the locus controlling this trait by heterozygosity mapping of partially congeneric lines carrying the mutagenized chromosome outcrossed to different inbred strains for three generations. Analysis of 68 PCR-based markers in 13 affected individuals indicated that the mutant locus, named Wheels (Whl), resides in the subcentromeric portion of mouse chromosome 4. The statistical evaluation of data obtained by heterozygosity mapping validates this efficient mapping approach. Further characterization of the Whl mutation demonstrated that Whl/Whl homozygotes die during embryonic life and that the penetrance of circling behavior depends on genetic background. Morphological analysis of the inner ears of Whl/+ mice revealed a variable number of abnormalities in the sensory and nonsensory portions of their semicircular canals. Abnormalities ranged from slight atrophy of one or more cristae to complete absence of the lateral crista and canal. The molecular characterization of the gene disrupted in the Whl mutation will provide insight into developmental mechanisms involved in inner ear formation.

Mutations associated with circling or waltzing behavior are remarkably common in the mouse (Deol 1966; Green 1989). Although the predominance of the circling/waltzing phenotype observed in mutagenesis screens as well as among spontaneously occurring mutations can be accounted for by its ease of recognition, it is equally likely that its prevalence may reflect the existence of a multitude of genes causing such a defect.

The majority of mouse mutations that exhibit a circling phenotype are associated with developmental anomalies leading to inner ear defects (Deol 1968; Steel et al. 1983). However, it is likely that both circling and inner ear abnormalities have a common (upstream) origin in the developing central nervous system, rather than the waltzing behavior being a consequence of the inner ear defect (Deol 1966; Steel and Bock 1985). Waltzing mutations display a wide range of severity and can reveal a recessive, semidominant or dominant mode of inheritance. These mutations are often pleiotropic or associated with other abnormalities such as pigmentation defects, cleft lip and palate, or more severe developmental defects leading to embryonic lethality (Deol 1968; Lyon and Searle 1989; Steel and Bock 1985). For example, a recent study of the mouse kreisler (kr) mutation showed that kr is involved in hindbrain segmentation. The kr/kr mice are characterized by circling behavior and severe malformation of the entire membranous labyrinth, while kr/kr embryos lack two rhombomeres (McKay et al. 1994; Cordes and Barsh 1994). Not all circling/waltzing mutations are accompanied by structural abnormalities of the inner ear. A targeted disruption of the brain-derived neurotrophic factor (BDNF) gene results in degeneration of the vestibular ganglion while the gross structure of the inner ear is normal (Ernfors et al. 1994; Jones et al. 1994). An inserional mutant, chakragati (ckr), with a recessive mode of inheritance, does not have any detectable inner ear anomaly. In this latter case, it is believed that the circling and movement disorders are both caused by a neurochemical dysfunction (Ratty et al. 1990; Fitzgerald et al. 1993).}

Despite our insight into the morphological aspects of inner ear development and the nature of structural anomalies sometimes associated with circling behavior, our understanding of the molecular mechanisms underlying neural control of inner ear differentiation is hindered by the paucity of genes known to be involved there. Nevertheless, in vertebrates, a subset of genes controlling basic embryo patterning, including Hox, FGF, TGF-β and HMG box cluster members, has been
shown to be expressed in developing ear structures (Pelton et al. 1991; Represa et al. 1991; Mansour et al. 1993; Mark et al. 1993; Oosterwegel et al. 1993; and for a review, Corey and Breakefield 1994). Consequently, our understanding of the entire developmental pathway might benefit from the isolation of genes defined by existing mouse mutations affecting ear development as well those associated with the waltzing phenotype.

In the case of insertional mutants, a transgene may provide an anchor in the search for the disrupted gene causing abnormal development/behavior (for a review, Meisler 1992). However, transgene insertions, similar to mouse mutations induced by irradiation, are often associated with large chromosomal rearrangements and the overall phenotype may be a consequence of the disruption of more than one gene (for a review, Davenport and Lewis 1990). Among chemically induced mutations, those induced by the potent mutagen N-ethyl-N-nitrosourea (ENU), are especially valuable due to the fact that the mutant phenotype, regardless of its complexity, is usually due to a point mutation in a single gene (for a review, Rinchik 1991). Recent advances in techniques of positional cloning, in particular approaches for the efficient identification of transcribed sequences, and the detection of single nucleotide substitutions by sequencing, facilitate the identification of any gene responsible for an ENU-induced mutation event.

We have identified an ENU-induced mouse mutation characterized by a complex neurological/behavioral phenotype including hyperactive circling/spinning, lengthened circadian period, and abnormal responses to light (G. E. Pickard, P. J. Sollars, E. M. Rinchik, P. M. Nolan and M. Bučan, unpublished work). To facilitate the genetic characterization and mapping of this mutation, we followed the inheritance of its most prominent mutant phenotype—circling/spinning behavior—in a series of intersubspecific and intraspecific crosses. In this paper, we describe mapping of the locus, named Wheels (Whl), causing hyperactive circling in heterozygotes and embryonic lethality in homozygotes, to the centromeric portion of mouse chromosome 4. The penetrance of circling behavior in heterozygote animals depends on the genetic background, and ranges from 25% (on a hybrid background) to 100% (on a C57BL/6J background). Likewise, the pathological changes identified in the inner ears of the mutants were quite variable, both across animals and in an individual animal’s right and left ears. These abnormalities ranged from slight atrophy of the crista in one or more semicircular canals (in behaviorally normal heterozygotes) to complete absence of the lateral semicircular canal and crista (in some rapidly circling animals).

The chromosomal localization of the Whl locus was determined by heterozygosity mapping of several partially congenic lines. We provide the statistical evaluation of the mapping data obtained by this efficient mapping approach suitable even for the localization of mutant loci associated with a complex phenotype and low penetrance.

**MATERIALS AND METHODS**

**Mice and genetic crosses:** The founder mouse (male 187) was identified in a behavioral screen among the progeny of BALB/c (mutagenized) males and (C57BL/10RI X C3H/RI)F females (G. E. Pickard, P. J. Sollars, E. M. Rinchik, P. M. Nolan and M. Bučan, unpublished work). To characterize abnormal circadian behavior, the founder mouse was outcrossed to C57BL/6J for two generations. For the genome scan, 13 affected progeny (generation 3—G3) of this backcross were used. In addition, mouse 187 was mated with several Mus musculus castaneus (CAST/Ei) females. Two F1 females from this cross, were mated with C57BL/6J males. Fifty-three backcross progeny were collected, from which DNA was isolated from tail tips and used for mapping studies. The progeny from this backcross carrying the Whl+/+ gene, or the BALB/cj and C57BL/6J alleles at the D4Mit181 and D4Mit149 loci, were used to Whl+/+ intercrosses to test the viability of Whl+/+ homozygotes. The examination of inner ears was performed on Whl+/+ mice from the outcross to the C57BL/6J inbred line, on C57BL/6J inbred mice (as a control) and on progeny from a Mus m. castaneus backcross, which included Whl+/+(circlers), Whl+/(noncirclers) and +/+(litter mates).

The inbred strains, C57BL/6J, BALB/cj, C3H/HeJ and CAST/Ei were purchased from the Jackson Laboratory, Bar Harbor, ME.

**Microsatellite markers:** All primer pairs for the genome scan were purchased from Research Genetics Inc. Listed by chromosome, they are as follows: chromosome 1: D1Mit30, D1Mit22, D1Mit26, D1Mit33, D1Mit17; chromosome 2: D2Mit83, D2Mit37, D2Mit62, D2Mit3, D2Mit48, (D2Mit1); chromosome 3: D3Mit94, D3Mit97, D3Mit10, D3Mit89; chromosome 4: D4Mit97, D4Mit81, D4Mit123, D4Mit64, (D4Mit49, D4Mit181, D4Mit1, D4Mit105); chromosome 5: D5Mit48, D5Mit108, D5Mit81, D5Mit4, D5Mit98, (D5Mit91); chromosome 6: D6Mit93, D6Mit4, D6Mit110, D6Mit25, (D6Mit104); chromosome 7: D7Mit77, D7Mit25, D7Mit62, D7Mit38, D7Mit101, (D7Mit21, D7Mit46); chromosome 8: D8Mit4, D8Mit27, D8Mit84, D8Mit14, chromosome 9: D9Mit91, D9Mit22, D9Mit10, D9Mit121; chromosome 10: D10Mit2, D10Mit15, D10Mit70, D10Mit14; chromosome 11: D11Mit1, D11Mit110, D11Mit8, D11Mit50; chromosome 12: D12Mit63, D12Mit101; chromosome 13: D13Mit17, D13Mit26, D13Mit78; chromosome 14: D14Mit14, D14Mit28, D14Mit75; chromosome 15: D15Mit58, D15Mit34, chromosome 16: D16Mit34, D16Mit5; chromosome 17: D17Mit21, D17Mit7; chromosome 18: D18Mit14, D18Mit19, D18Mit3; chromosome 19: D19Mit21, D19Mit7. Primer pairs used for additional mapping and genotyping are indicated in parentheses. Each microsatellite primer pair was initially analyzed in DNA isolated from the founder mouse and F1 progeny between C57BL/6J and BALB/cj mice to test whether both alleles are efficiently PCR amplified.

**Isolation of DNA:** Mouse tail DNA was prepared as follows: part of a tail (1–2 cm in length) was incubated overnight at 55°C in 700 µl of lysis solution (50 mM Tris, pH 8.0; 100 mM EDTA; 0.5% SDS; 350 µg proteinase K). Genomic DNA was phenol/chloroform extracted and precipitated by ethanol (Hogan et al. 1986).

**Microsatellite analysis:** High molecular weight tail DNA was used as template for PCR reactions. The single-strand length polymorphisms (SSLP) identified by microsatellite markers were detected using a slightly modified version of the protocol described by Dieterich and co-workers (1992). PCR reaction...
mixtures included 50-ng template genomic DNA, 100 μM dNTPs, 0.5 μCi 32P-α-dCTP (Amersham, Arlington Heights, IL), 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl2, 1.65 pmol of the forward, 1.65 pmol of the reverse primer and 1 u Ampli Taq DNA Polymerase (Perkin-Elmer-Cetus, Emeryville, CA). PCR conditions were as follows: 35 cycles of 1 min at 94°, 2 min at 55° and 2 min at 72°, followed by one cycle of 7 min at 72°. The denatured PCR products were run on a 6% polyacrylamide, 8 M urea sequencing gel, and polymeric bands visualized by autoradiography.

**Microscopic examination of inner ear structures:** Thirteen mice were prepared for microscopic examination of their inner ears using a modification of our usual cochlear preparation techniques (Bohne 1972; Bohne and Harding 1993). After deep anesthesia, the mice were perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde in phosphate buffer. The animals were decapitated and their heads immered in a large volume of fixative for several days. After initial fixation, each head was washed in two 1-hr changes of phosphate buffer. The bullae were opened to reveal the middle and inner ears. Using a sharpened pick, small perfusion holes were made in the bone at the cochlear apex and over the middle of the superior semicircular canal. The stapes was separated from its attachment to the oval window. The second fixative [1% buffered osmium tetroxide (OsO4)] was gently circulated through the perilymphatic spaces of the inner ear after which the specimens were immersed in additional fixative for 1 hr. After washing in buffer (three times, 15 min each), the temporal bones were cleaned of all adhering soft tissue and the facial nerve was removed from its canal. Infiltration holes were made in the apex and base of the cochlea. The specimens were dehydrated in a graded series of ethanol and propylene oxide and gradually infiltrated with Durcupan (epoxy resin). After being dehydrated and infiltrated with plastic, the temporal bones became quite transparent. At this point, the OsO4-stained membranous labyrinth was inspected in its entirety for any gross malformations while the specimen was immersed in liquid plastic. The specimens were then placed in fresh Durcupan which was polymerized at 60° for 48 hr. Razor blades were used to trim away excess plastic from outside the temporal bone. A sharpened, steel pick was used to carefully chip away the bone over the membranous labyrinth. Small pieces of razor blades were also used to remove half-turns of the cochlear duct from cochlear apex to base and to separate the five vestibular sensory areas from each other. The plastic in the perilymphatic spaces of the cochlea was trimmed close to the basilar membrane while the cristae and maculae were hand cut at a radial angle into several pieces. After reembedding in 2-mm thick blocks of plastic, the sensory epithelia of the auditory and vestibular systems were examined as whole mounts by phase-contrast microscopy.

**RESULTS**

**General observations and behavioral anomalies:** The *Wheels* mutation (Whl) was initially discovered in a behavioral screen for mice exhibiting altered circadian rhythms performed by monitoring locomotor activity (wheel-running activity) in constant dark conditions. Among ~300 progeny of mutagenized males (BALB/cRl) and (C57BL/10Rl X C3HFl/Rl)Fl females, we identified one animal (187) with an activity period significantly longer than normal (G. E. Pickard, J. P. Sollars, E. M. Rinchik, P. M. Nolan and M. Bučan, unpublished work). However, in addition to altered circadian period, the founder mouse exhibited bidirectional circling behavior. Circling and abnormal period were transmitted to the next generation, although both mutant phenotypes were expressed in fewer animals than predicted for a dominant mutation and it was not clear whether the overall mutant phenotype was due to a single gene defect.

Aside from behavioral anomalies (circling/spinning, lengthened circadian period, hyperactivity) mutant mice were smaller in size than their littermates and, with age, some of the affected animals displayed a hunched stance and/or ataxia. Mutant mice can be startled by sudden sounds and exhibit normal swimming behavior. These observations indicate that the mice have some hearing ability and vestibular function. Also, some circling animals (one out of 10) display symmetrical or asymmetrical reduction of eye size. Although both sexes are fertile, affected (circling) mothers do not care well for their litters and the survival rate of pups is dramatically improved by crosses between affected males and wild-type females. However, the ratio of affected (circling) to normal animals in a large number of G2 and G3 litters suggests that some affected animals die either in utero or around weaning. The circling behavior is first apparent at 2 weeks of age, however, at that time it is not accompanied by hyperactivity. Although adult mice are hyperactive during the periods of circling and spinning, they do not appear to be incessantly hyperactive.

The complex behavioral phenotype, possible incomplete penetrance and variable expressivity, as well as compromised survival rate of heterozygotes forced us to initially focus on only the most prominent mutant phenotype (circling) in the characterization of the mutant locus. We reasoned that the genetic localization of a mutant locus controlling circling may provide a means of genotyping animals that will be tested in more complex behavioral paradigms and that the results of genotyping may facilitate the interpretation of behavioral data.

**Heterozygosity mapping of partially congenic lines:** To rapidly identify the chromosomal region that could potentially carry the mutation causing circling, we applied a mapping strategy similar to one used for the homozygosity mapping of recessive traits in affected children of related parents (Lander and Botstein 1987). We made use of 13 affected animals obtained by outbreeding the mutant line 187 to a C57BL/6j inbred strain for two generations. The original ENU mutagenesis was carried out in BALB/cRI males, which were mated once with (C57BL/10Rl X C3HFl/Rl)Fl females. One of their progeny, the founder mouse (187), was subsequently outcrossed to C57BL/6j for two generations (Figure 1A). Successive outbreeding to the C57BL/6j background, in parallel with the selection for the mutant phenotype, would eventually generate a congenic line of mice with a C57BL/6j background and BALB/c-chromosome derived DNA around the
mutant locus. In our experiment, we took advantage of this partially congenic line arguing that the genome of 13 affected mice used for mapping will theoretically contain 12.5% of BALB/c DNA scattered throughout the mouse genome, but that BALB/c/C57BL/6J heterozygosity should be consistently detected at and around the mutant locus of all affected animals.

We prepared DNA from the tails of 13 G3 animals that expressed the circling phenotype. Among the large number of available SSLP microsatellite markers, we chose 68 primer pairs that amplify sequences (dispersed over the entire autosomal portion of the genome) that are polymorphic for BALB/c, C57BL/6J and C57H/He DNA. This number should allow us to detect linkage within ~95% of the mouse genome. As expected, the percentage of BALB/c specific alleles detected in the 13 G3 circlers ranged from 8 to 19% (with an average percentage of 14.1). Figure 1B shows a representative example of a marker (D10Mit15) that detects BALB/c and C57BL/6J alleles in eight animals and C57BL/6J homozygosity in five animals, a microsatellite marker (D3Mit89) that maps to the chromosomal segment homozygous for the C57BL/6J chromosome in all 13 animals, and D4Mit105 which represents a marker from the chromosomal region that retained BALBc/C57BL/6J heterozygosity in 12 out of 13 animals tested. Of all 68 markers tested in the genome scan, only one marker showed more than 75% BALB/c/C57BL/6J heterozygosity (Figure 2). This marker (D4Mit105) was previously mapped to the subcentromeric region of chromosome 4 (Figure 3; Whitehead Institute/MIT Center for Genome Research, updated list of SSLP loci in the mouse).

Statistical analysis of data obtained by heterozygosity mapping: The statistical analysis consists of two stages, which we discuss in turn. The first stage relates to individual marker loci and focuses, for each marker locus, on the number of heterozygous (BALB/c/C57BL/6J) mice in the sample. Probabilities associated with the number of heterozygous mice in the sample cannot be calculated directly using the binomial distribution formula, but must take account of the ancestral pattern of mice in the sample. We calculate here only the required probabilities for the ancestral pattern of our own sample (see Figure 4), leaving a more general discussion to the APPENDIX.

Consider a hypothetical marker unlinked to the Whl locus. All 13 sampled mice (in generation 3) will be
heterozygous at this marker locus only if both the (generation 2) parents are heterozygous (probability $1/4$) and then, given this, that all 13 sampled mice are heterozygous [probability $(1/2)^{13}$], leading to a final probability of $(1/2)^{15} = 0.0000305$. Similarly, 12 of the sampled mice will be heterozygous only if both parents are heterozygous, and then given this, that 12 of the 13 sampled mice are heterozygous is thus $0.0004272$. Probabilities for 2, 11, 210 and 29 heterozygous mice in the sample can be calculated in a similar way, the probability for nine or more heterozygous mice being 0.0334. No other probabilities of this nature are <0.05.

In our data, we observe one locus (D4Mit105) with 12 heterozygous mice, and the above calculation shows that, for the moment, this is a highly significant value. We also observed a locus (D2Mit37), with nine heterozygous mice, for the moment a marginally significant value (Type I error 5%). These are the only two potentially significant marker loci, that is, the only two marker loci that we consider as candidates for being linked to Whl.

This brings us to the second stage of the statistical analysis. Here we take account of the fact that 68 marker loci were tested, so that even if no markers are linked to the Whl locus, we would expect, using a Type I error of 5% for each locus, to find approximately three or four significant results simply by chance. Correcting for multiple markers is not easy, because tests for markers on the same chromosome are not independent. The assumption of independent markers, however, provides a conservative test, and this gives a conservative significance level of $1 - (0.99957)^{68} = 0.0286$ for the D4Mit105 locus, significant at the 5% level. A non-conservative test arises by assuming complete linkage of all markers on the same chromosome, and this test ascribes a non-conservative significance level of $1 - (0.9666)^{19} = 0.48$ for the D2Mit37 locus, nowhere near significant. We thus assess that the only marker locus closely linked to Whl is D4Mit105.
Significance may be difficult to achieve, in general, with the conservative test, especially in small samples, so that a reasonable procedure is to retest those marker loci which are close to significance, using new data. Although this did not occur in our case, we anticipated this problem and set aside an independent sample of 10 mice for further testing. All 10 mice were heterozygous at the D4Mit105 locus (probability 0.001), confirming our assessment of linkage of this locus to Whl, while no other marker locus (e.g., D2Mit37) was significant, (again confirming our conclusion from the original 13 mice). In addition, the location for the Whl locus was subsequently confirmed by the finding that two additional SSLP markers, D4Mit149 and D4Mit181, detected heterozygosity in all 13, originally tested, affected G3 animals, while 44 phenotypically normal G3 progeny did not reveal the BALB/c allele using either marker. The final result is that the Whl locus maps to the subcentromeric portion of mouse chromosome 4.

The Whl locus maps within the chromosomal segment spanning 8 cM distal to the centromere of chromosome 4 as determined on the microsatellite map (Whitehead Institute/MIT Center for Genome Research, updated list of SSLP loci in the mouse; Figure 3). The loci D4Mit149 and D4Mit181, mapped within the 3.3-cM region distal to the centromere, detected BALB/c/C57BL/6J heterozygosity in every mouse exhibiting circling behavior. The D4Mit1 marker, located 8 cM distal to the centromere, identified two recombinants—C57BL/6J homozygosity in circling animals among >20 tested so far. To date, three genes have been localized within the Whl candidate region: the proto-oncogene, Mos; a member of the Src family of tyrosine kinases, Lyn; and a CNS-specific POU transcription factor, Otf7 (Bra2) ( Abbott et al. 1993).

The influence of genetic background on severity of Whl mutation: The genome scan provided the chromosomal localization of the Whl locus and indicated that circling is a fully penetrant mutant phenotype in mice outcrossed to the C57BL/6J background for five generations. We have also followed the inheritance of circling behavior in an intersubspecific backcross involving the evolutionary distant strain Mus m. castaneus. For this purpose, the founder mouse 187 was mated with several Mus m. castaneus females. It is interesting that two F1 females that did not exhibit circling behavior (although one of them would occasionally perform somersaults) gave birth to circling progeny when backcrossed to C57BL/6J. This finding provided an indication that, in a hybrid background, the circling phenotype is not completely penetrant. The genotyping of backcross progeny at the D4Mit181 locus, by following the segregation of the BALB/c allele, allowed us to evaluate the penetrance of circling behavior in this interspecific backcross. Among 53 N2 animals tested so far, only seven circling progeny have been identified whereas the BALB/c allele at the D4Mit181 and D4Mit149 loci was detected in 28 out of 53 animals, indicating that penetrance of the circling phenotype in the progeny of a Mus m. castaneus backcross is extremely low (25%).

Homoygote lethality of Whl/Whl: In an attempt to determine whether Whl/Whl homozygous mice are viable, we collected 28 newborn pups from a Whl/+ intercross on the first postnatal day and investigated by PCR genotyping whether any Whl/Whl mice survive until birth. PCR analysis of tail DNA with the D4Mit149 or D4Mit181 markers did not detect BALB/c homozygosity, confirming our preliminary observation that the Whl locus is associated with embryonic lethality.

Histopathological examination of the inner ear: We histologically examined ears of several C57BL/6J inbred mice and of 13 mice—circling and noncircling G2 and G3 progeny from the following crosses: Whl/C57BL/6J × C57BL/6J and Whl/Mus m. castaneus × C57BL/6J. Based on the presence and absence of BALB/c DNA at the D4Mit149 and D4Mit181 loci, tested backcross progeny were grouped into controls (+/+), circlers (Whl/+, n = 4) and carriers (Whl/+, n = 2)—mice that did contain a mutant gene but did not express it behaviorally.

The auditory portion of the inner ear was entirely normal (Figures 5 and 6) in all three genotypes, including the density of sensory cells within the organ of Corti. In the +/+ animals, the sensory and nonsensory areas of the utricle, saccule and semicircular canals had morphological appearances (Figure 5, A and B) as typically described (e.g., Hunter-duvar and Hinojoa 1984). Defects in the vestibular apparatus of Whl/+ circlers were variable both in an individual mouse’s left and right ears and in different animals. All circlers had at least one ear in which the lateral semicircular canal was incomplete or absent (Figure 5, C and D). In addition, all but one circler had one or more cristae, which were atrophic or absent. Defects in the lateral crista were most frequent, followed by those in the posterior and then the superior cristae. Defects in the Whl/+ carriers consisted of atrophy of one or more cristae (Figure 6). None of the carriers had an incomplete lateral canal.

DISCUSSION

We have mapped a novel neurological mutation, Whl, to the subcentromeric region of mouse chromosome 4. Rapid bidirectional circling is the most overt behavior in Whl/+ mice, and this could be due to the varying degree of structural anomalies of the inner ear, in particular, the lateral semicircular canals and cristae. The genetic analysis of the Whl locus and identification of markers that can be used for genotyping provide an essential tool in the further characterization of a variety of interesting behavioral anomalies initially observed in Whl/+ mice (G. E. Pickard, P. J. Sollars, E. M. Rinchik, P. M. Nolan and M. Bucan, unpublished work). The pleiotropic effect of the Whl mutation and a
**FIGURE 5.**—Inner ears from two wild-type mice are shown in A and B while C and D illustrate the left and right ears, respectively, from a Wh/+ mouse that exhibited circling behavior. C, cochlear duct; OW, oval window; RW, round window. (A) Three semicircular canals, lateral (LSC), posterior (PSC), superior (SSC), can be seen through the transparent bone. (B) Four of five sensory areas in the vestibule are visible: LC (lateral crista), MU (utricular macula), PC (posterior crista) and SC (superior crista). Nerve to posterior crista (N. PC) is visible through the RW. Magnification bar on A also applies to B. (C) In left ear, lateral (LC) and superior (SC) cristae are visible along with nerve to posterior crista (N. PC). No remnant of the lateral canal is present. (D) In right ear, posterior (PC) and superior (SC) cristae and utricular macula (MU) are visible but the lateral canal and crista are missing. Magnification bar on C also applies to D. In C and D, white arrows mark the approximate location of the missing lateral canal.
suppressed mutant phenotype (circling) in F1 progeny between Whl/+ and Mus m. castaneus mice hindered the generation of a conventional linkage cross suitable for the chromosomal mapping of the Whl locus. Therefore we mapped the Whl locus using a whole genome search for the chromosomal segment (marked by BALB/c specific alleles of SSLP loci) bearing the mutant locus in several affected (circling) partially congenic lines (Figure 1). This efficient and rapid approach has points of similarity with the method described by LANDER and BOTSTEIN (1987). Our modification, applicable only for the analysis of controlled matings, is particularly convenient for the initial characterization of new behavioral (semi) dominant mutations associated with pleiotropic effects and incomplete penetrance. However, this procedure can easily be adapted for the analysis of recessive traits. A small amount of sample material—DNA isolated from the tail tips of a few-week-old animals—is sufficient to determine the chromosomal localization of a mutant locus, while animals can be further subjected to a variety of behavioral tests. Although the application of partially congenic lines to determine a provisional chromosomal location has been used in mouse genetics for a long time, this method is currently particularly attractive in conjunction with the availability of a large number of PCR based DNA markers—polymorphic microsatellite loci (DIETRICH et al. 1992).

In the course of the mapping of the Whl mutation, we have assumed that the mutation was induced by ENU mutagenesis (on a BALB/c chromosome) and does not represent a spontaneous mutation event that happened in the maternal genome [(C57BL/10RI × C3Hf/R1) F1]. In the latter case we would not be able to determine the chromosomal location using a genome scan to search for the chromosomal region associated with the BALB/c/C57BL/6 heterozygosity. The transfer of a mutant locus by successive outcrossing from one genetic background to another, or from a heterogeneous to inbred background, is often used in the analysis of behavioral mutations because of the profound effect of genetic background on the expressivity or penetrance of the mutant phenotype. To narrow down the location of the Whl locus to ~1 cM—a genetic distance suitable for the initiation of a positional cloning effort—it will be necessary to generate a conventional high-resolution linkage stock.

The Whl locus maps within the 8-cM region distal to the centromere on chromosome 4. This map location allowed us to rule out the possibility that the Whl mutation may be allelic to several dominant circling muta-
tions already identified in mutagenesis experiments or those arose as spontaneous mutations (GREEN 1989). Transgenic mice overexpressing the protooncogene Mos, a gene that maps to the Whl candidate region, exhibit hyperactive circling behavior similar to Whl/+ mutants (PROST et al. 1990). However, Mos transgenics are also ataxic, tilt their heads and bodies at rest and in motion and have no startle response or brain stem auditory evoked potentials. Histopathological studies have shown that Mos transgenics have generalized neuronal and axonal degeneration, gliosis and inflammatory infiltrates in the CNS that have not been seen in Whl/+ mice (K. MONTONE, personal communication). Although the appearance of the entire membranous labyrinth in Mos transgenics has not been described, one would expect abnormalities in some vestibular organs, based on the behavioral observations. Mos transgenics are missing many cochlear sensory and supporting cells and some spiral ganglion cells, thus accounting for their deafness. In Whl/+ mice, inner ear abnormalities are confined to the vestibular division of the inner ear.

The inner ear abnormalities observed in the Whl mice are not unique. Most so-called “shaker-waltzer” mutants have abnormalities in the vestibular portion of their inner ears (STEEL et al. 1983), although the behavioral manifestations may be the result of defects in the CNS rather than the peripheral vestibular organs (DEOL 1966). Heterozygotes for the Twirler (Tw) mutation (LYON 1958) and an insertion mutation (Tg9257) mapped close to the Tw locus (TING et al. 1994) exhibit circling behavior and have a shortened or absent horizontal semicircular canal and a flattened or inverted horizontal crista. Although these mutations have a certain similarity with Whl, the former mutations also have craniofacial malformations such as hypoplastic facial bones or cleft palate. The presence of facial malformations indicates that the molecular mechanism leading to the abnormal phenotypes is distinct for Tw, Tg9257 and Whl.

Similar to the majority of dominant mutations causing the shaker/waltzing phenotype, the Whl mutation is associated with embryonic lethality in homozygotes. Genotyping of embryos at several gestational stages will allow us to determine the exact time of death during intrauterine life, and the morphological examination of mutant embryos may provide a clue to the nature of the primary defect caused by the mutation. The newborn Whl/+ pups are smaller at birth than their littermates. This observation suggests that growth retardation is caused by a developmental defect, and is independent of hyperactivity, which has a late onset (later than the onset of circling behavior).

In humans, abnormalities of the horizontal (lateral) canals represent the most common defect in congenital inner ear anomalies and may lead to disequilibrium (SANDO et al. 1988). The map location of the Whl locus in the subcentromeric region of mouse chromosome 4 allows the prediction, based on the existing comparative map, that the locus homologous to Whl maps on human chromosome 8. The Mos and Lyn loci have been mapped to 8q11-q13 (CAUBET et al. 1985), however, no neurological disorders similar to Whl have been reported in association with this chromosomal region.

The Whl mutation was identified among the progeny of ENU-treated animals. The ENU-induced mutations are most frequently associated with point mutations rather than deletions or large chromosomal rearrangements (VOGEL and NATARAJAN 1979; POPP et al. 1983). The Whl gene product may be a key developmental regulator whose haplo-insufficiency causes developmental defects. However, the semidominant nature of the Whl mutation can also be due to a mutation event causing a changed expression pattern (gain of function) or interference with the function of the wild-type allele, giving a dominant-negative effect. These questions, as well as the variable expressivity of the mutant phenotypes and the origin of the left-right asymmetry of inner ear abnormalities will be elucidated only by identification and expression analysis of a mutant gene.

The Whl mutation described in this report adds to the value of the existing developmental mutations affecting inner ear development. Moreover, the fact that the founder mouse exhibited abnormal circadian behavior and response to light indicates that the further analysis of the molecular mechanism underlying the mutant phenotype may provide insight into more general neurodevelopmental events.

We would like to thank G. RINCHIK, V. CHAPMAN, J. NADADEV and S. POETHING for helpful discussions, P. WHITROW and G. GOTTLEB for their support, J. TRAN, T. J. WATKINS and R. L. BURMEISTER for technical assistance, and N. MCCULL and K. GALLO for animal care. These studies were supported by the Center for Sleep and Respiratory Neurobiology at the University of Pennsylvania, National Institute of Mental Health Grant MH-47501 to G.E.P., National Institute of General Medical Sciences Grant GM-21135 to W.E., a National Institute of Deafness and Other Communications Disorders Grant DC-00671 to B.A.B, a grant from the Air Force Office for Scientific Research (F49620-94-1-0234) and the Research Foundation of the University of Pennsylvania to M.B.

LITERATURE CITED


BOHNE, B. A., 1972 Location of small cochlear lesions by phase contrast microscopy prior to thin sectioning. Laryngoscope 82: 1–16.


DAVISSON, M. T., and S. E. LEWIS, 1990 Chromosome aberrations


Communicating editor: K. ARTZT

APPENDIX

We provide, in this appendix, generalizations of the statistical analysis given above for our data.

Suppose in general that a sample of n mice is taken g generations after the initial mutant. The direct generalization of our results is that the probability that all n mice in the sample are heterozygous at an unlinked marker locus is \( (1/2)^n \), where a is the total number of distinct ancestors in generations 2, 3, ..., g - 1 of the sampled mice. In our case, \( n = 13 \), a = 2 and the above is our probability \( (1/2)^{15} \). The probability that \( n - 1 \) mice are heterozygous is \( n(1/2)^{n+a} \), again leading to a probability calculated above. These calculations are sufficient (as they were in our case) to provide probabilities for marker loci with \( n - 1 \) or \( n \) heterozygous mice.

For simple ancestral patterns these calculations can easily be made for \( n - 2, n - 3, \) and fewer mice, as in our case. For more complex ancestral patterns, these calculations rapidly become complicated, and approximating methods might be needed. For an unlinked marker, the mean M of the number of heterozygous mice in the sample at this locus is \( n(1/2)^{-1} \), whatever the ancestry pattern. The variance V of the number of heterozygous mice depends on the ancestry pattern and can be shown to be given by

\[
V = n(1/2)^{-1} + \frac{1}{2} \sum (1/2)^{n(i,j)} - n^2(1/2)^{2}\epsilon^{-2}.
\]

Here the double summation is taken over all pairs of mice in the sample, and \( m(i,j) \) is the total number of distinct ancestors, in generations 2, 3, ..., \( g - 1 \), of mice i and j. An approximate significance level can be found by using M and V and assuming a normal distribution for the number of heterozygous mice, although the exact distribution of this number can be quite skewed, so this approximation should be used with caution.

Generalizations of this theory to cover the case of recessive mutations can also be calculated (W. J. EWENS, P. M. NOLAN and M. BUCAN, unpublished data).