A hearing and vestibular phenotyping pipeline to identify mouse mutants with hearing impairment

Rachel E Hardisty-Hughes, Andrew Parker & Steve D M Brown

MRC Harwell, Mammalian Genetics Unit, Harwell Science and Innovation Campus, Harwell, UK. Correspondence should be addressed to S.D.M.B. (s.brown@har.mrc.ac.uk).

Published online 7 January 2010; doi:10.1038/nprot.2009.204

We describe a protocol for the production of mice carrying *N*-ethyl-*N*-nitrosourea (ENU) mutations and their screening for auditory and vestibular phenotypes. In comparison with the procedures describing individual phenotyping tests, this protocol integrates a set of tests for the comprehensive determination of the causes of hearing loss. It comprises a primary screen of relatively simple auditory and vestibular tests. A variety of secondary phenotyping protocols are also described for further investigating the deaf and vestibular mutants identified in the primary screen. The screen can be applied to potentially thousands of mutant mice, produced either by ENU or other mutagenesis approaches. Primary screening protocols take no longer than a few minutes, apart from ABR testing which takes upto 3.5 h per mouse. These protocols have been applied for the identification of mouse models of human deafness and are a key component for investigating the genes and genetic pathways involved in hereditary deafness.

INTRODUCTION

The mouse offers an efficient tool kit for determining gene function in the mammalian genome and, as a consequence, has proved to be one of the key model organisms for functional genomics studies[1]. For example, the mouse has made a significant contribution to understanding the genetics of hearing impairment[2]. The similarities in the structure and physiology of the human and mouse ears along with the close evolutionary relationships between the two genomes makes the mouse a crucial model system for the study of functional genomics of the auditory system. The complexity of the hearing and vestibular apparatus is reflected in the genetic repertoire that is responsible for its development and function. Nearly 130 loci that cause human deafness have been mapped, but many genes have not been identified and we are far from determining a complete picture of the genetic networks underlying the auditory system (http://webh01.ua.ac.be/hhh).

One of the major approaches for the assessment of gene function is random chemical mutagenesis of the mouse genome in a phenotype to gene approach (the so-called phenotype-driven or forward genetics approach)[3,4]. This involves screening of mice for mutant phenotypes without previous knowledge of the genetic basis of the mutation. The chemical mutagen of choice for this approach is *N*-ethyl-*N*-nitrosourea (ENU). ENU phenotype-driven screens have been carried out for a number of disease systems. Several major ENU programs have applied a variety of hearing and vestibular phenotype tests, and these have been extremely effective in identifying a wide variety of deafness models[2]. The development of phenotype-driven screens for auditory phenotypes has mirrored a wider effort across the mouse genetics community to develop and apply efficient phenotyping pipelines for a variety of disease systems[1,4]. ENU mutagenesis has been successful in uncovering disease models for bone[5,6], neurological[7], vision[8,9], metabolic[10,11] and behavioral disorders[12] amongst others.

This protocol aims at providing a detailed description of primary and secondary phenotyping tests that we use in our laboratory to allow mutant mice with hearing impairment to be identified. It is important to note that these protocols, while being used by us principally for the analysis of ENU mutant mice, can also be utilized for the characterization of any genetically altered mouse line or for inbred and outbred strains of mice. For example, the protocol can be applied to mouse mutants produced by other mutagenesis approaches including gene targeting, gene trapping and transposon mutagenesis[13].

In determining the phenotypes for an organ system, individual tests are commonly used, often on an *ad hoc* basis. However, it is increasingly being recognized that it is more efficient and provides a more comprehensive description of the phenotyping outcomes and the underlying pathophysiology of a mutation, to employ a battery of tests[1]. Such phenotyping pipelines are increasingly being used to identify and describe mouse models of human disease. Many individual tests are utilized for assessing the auditory and vestibular systems of mice. In contrast, we have developed an auditory and vestibular phenotyping pipeline, comprising a battery of tests that we employ for a broad characterization of the auditory and vestibular phenotypes of mouse mutants. This phenotyping platform is designed to be used when a more systematic determination of the origins of hearing loss is required, thus leading to a more profound understanding of the underlying etiology. It is important to state that this phenotyping pipeline does not represent a comprehensive set of auditory phenotyping tests. For example, imaging modalities, such as optical projected tomography (OPT), represent new phenotyping platforms with considerable potential. OPT allows for 3D visualization of biological samples much larger than previously possible with techniques such as deconvolution, confocal microscopy or optical coherence tomography[14]. It provides a rapid route for an overall assessment of the cochlear structure but is not further used in this protocol. However, the pipeline represents a battery of essential tests from the simple to the more complex that can be used by the investigator to determine whether a mouse has an auditory and/or vestibular defect and the pathological nature of the defect. We consider this set of tests a minimum battery for an auditory and vestibular phenotyping pipeline. Moreover, we describe a pipeline where the tests are readily accessible and interpretable. Other more sophisticated tests are available, but they do not necessarily represent the analyses that would be carried out as a primary or essential initial characterization of auditory and vestibular function. For example, we have not included more

BOX 1 | DOMINANT AND RECESSIVE *N*-ETHYL-*N*-NITROSOUREA (ENU) MUTAGENESIS PIPELINES

In order to produce ENU mutant animals, male mice from an inbred strain are treated with ENU. Dose regimes for efficient mutagenesis have been established for a number of inbred strains, including C57BL/6, BALB/c and C3H. After a period of infertility, treated mice regain fertility and are mated to produce offspring carrying ENU mutations. Often, ENU-treated male mice are mated to mice of a different strain. This allows for rapid genetic mapping of any ENU mutation discovered in the progeny, as they will be segregating DNA variants from the different parental inbred strains that can be used for genetic analysis of the mutation and localization to a chromosome region. Sometimes, ENU-treated male mice are crossed to female mice from the same strain. However, in these circumstances, if a phenotype is discovered in the progeny, then the phenodeviant mouse must be outcrossed to another inbred strain for genetic mapping.

Dominant ENU mutagenesis pipelines. ENU-treated male mice are mated and the first generation (G1) progeny are screened for disease phenotypes. Given the specific locus mutation rate of ENU, it is usual to screen at minimum 1,000–2,000 G1 progeny to have a high probability of hitting any locus involved in the relevant disease pathway. Most G1 progeny will be normal with respect to the disease phenotypes under investigation and therefore, they act as a set of baseline controls.

Recessive ENU mutagenesis pipelines. ENU-treated male mice are mated and the male G1 progeny are mated again to produce G2 females that are backcrossed to the founder G1 male. From each G1 male, a number of G3 progeny are produced. Some mice from each pedigree (on average one in every eight) will be homozygous for mutations carried by the founder G1 male. Screens of G3 progeny will reveal recessive phenotypes associated with homozygous mutations from the male founder. Given the likelihood of homozygous mutations from the G1 founder male, around 30–40 mice are analyzed from each pedigree to establish the presence or absence of relevant disease phenotypes. The identification of two or more similar phenotypes within a pedigree is confirmation of a heritable recessive phenotype segregating within the pedigree. If the G1 and G2 progeny were generated by outcrossing, then the G3 progeny can be utilized directly for genetic mapping of the mutant phenotype. Most of the G3 progeny will be of wild-type phenotype and, as with dominant screens, act as a baseline control. Typically, 100 or more pedigrees might be analyzed to uncover recessive phenotypes and investigate loci involved with a particular disease system.

specialized auditory tests, such as distortion product otoacoustic emission (DPOAE), which can provide detailed functional information on outer hair cell (OHC) function[15].

It is important to note that each test can be used in isolation and provide information on vestibular, auditory or structural defects as indicated below. However, the outcomes of each test, whether positive or negative, together provide a phenotypic landscape of the auditory and vestibular system for each mutant analyzed. As such the results from the phenotyping pipeline are often a starting point for further more elaborate and invasive testing that will amplify and elaborate the mutant phenotype and the genetic etiology.

Experimental design

ENU mutagenesis. The basis and techniques of ENU mutagenesis (see **Box 1**) have been extensively reviewed[4]. ENU is a powerful chemical mutagen that is administered by a series of intraperitoneal injections into male mice, where it acts on spermatogonial stem cells. The dose and injection regimes for the most efficient mutagenesis vary between different inbred strains[16] (**Box 2**). Typical treatment regimes involve two or three rounds of injections, each a week apart, at a dose of 80–100 mg kg^{-1}. Male mice treated with ENU undergo a period of infertility and subsequent to regaining fertility, they are bred to generate progeny carrying ENU-induced mutations. ENU is an alkylating agent that introduces point mutations with a high specific locus mutation rate of around 0.0015 (ref. 4). There are two main approaches used to generate ENU mutagenized animals for auditory phenotyping: a G1 pipeline for dominant mutations and a G3 pipeline for recessive mutations (**Fig. 1a,b**). In the present mutagenesis pipeline used at MRC Harwell for hearing and vestibular screens, ENU mutagenized C57BL/6 animals are crossed to C3H/HeH to produce G1 progeny for the dominant screen. To generate G3 animals and screen for recessive phenotypes, G2 females are generated and backcrossed with the founder G1 male.

Vestibular and auditory phenotyping. Mice from both dominant and recessive ENU mutagenesis pipelines (see **Box 1**) enter hearing and vestibular phenotyping (**Fig. 2**). First, both G1 and G3 progeny enter a simple primary screen for vestibular and auditory function in the phenotyping pipeline (outlined on the website: http://empress.har.mrc.ac.uk) before more sophisticated primary and secondary investigations are undertaken (see **Fig. 2**). It is important at each stage of the phenotyping pipeline to utilize wild-type mice as controls. These could be inbred mice of the appropriate genetic background or wild-type littermates from the breeding colonies of the mutant mice.

BOX 2 | *N*-ETHYL-*N*-NITROSOUREA (ENU) MUTAGENESIS: 56 WEEKS

The strategy for the ENU dosing at MRC Harwell has been described[37]. In short, 8–10-weeks old C57BL/6 male mice are injected with 3 × 100 mg kg^{-1} body weight ENU, over a 3-week period. Mice are sterility tested by placing the mice in test matings for 12 weeks; effective mutagenesis is indicated by a period of sterility and the absence of offspring. After 12 weeks, those mice that do not produce offspring are used in the production pipelines (G0 males). Time from the first ENU injection to G0 males to testing G1 progeny is a total of 26 weeks (3 weeks for ENU injections, 12 weeks to sterility test, breeding of G0 mice to produce G1 offspring that are subsequently aged to 8 weeks to undergo primary testing). In order to produce G3 offspring (of testable age), G1 males are outcrossed producing G2 mice, which are then backcrossed to the G1 father. The production of G2 and G3 mice adds another 22 weeks to the protocol, assuming there is no delay in breeding.

Primary screening of mutant mice. *Tests for vestibular function*: As well as hearing loss, mice with a perturbation in the development and function of auditory (ear) structures often display a vestibular component to their abnormal phenotype. Though this behavior may be obvious (even to an untrained observer), it is, however, prudent to try and characterize and quantify this behavior, as this may give an indication of the primary site of the pathology (e.g., gravity receptors versus semi-circular canals) and we utilize a number of tests to assess vestibular dysfunction (**Fig. 3**). Initially, we observe mice for behavioral signs such as head tossing/bobbing[17] or **shaker–waltzer behavior** that are indicative of a vestibular disorder[18]. Subsequently, a simple **trunk curl** test is undertaken in which the ability of mice when held by the tail to reach out to a presented horizontal surface is gauged. Control mice will reach towards this landing surface, whereas mice with vestibular impairment classically curl towards their abdomen (*forward* curling), thus tending toward an occipital landing (**Fig. 3c,d**). Next, we carry out a **Contact righting** test in which mice are placed in a perspex tube and inverted. This test is designed to assess if animals will respond to pressure applied to the feet (by stimulation of proprioreceptors which sense body position) by walking along the newly introduced ventral surface[19]. Control animals with a normal vestibular apparatus will ignore this surface and rotate back to their upright position. However, animals with a lack of vestibular integrity would rely on proprioreceptive input alone for orientation and would remain inverted. We conclude vestibular testing with the **swim test**. Mice with vestibular dysfunction are often able to maintain normal posture in their home cages because of input from tactile and visual cues, even in the absence of vestibular information. In water, mice have to rely on vestibular information for orientation and the swimming test often reveals mutants with vestibular abnormality, which is otherwise not apparent[20]. Swimming is scored in the following way:

- 0 = Swims. Mouse body is elongated and the tail propels in a flagella-like motion.
- 1 = Irregular swimming. Includes: vertical swimming, swimming in a circle, swimming on side (left/right preference should be noted), swimming in an unbalanced manner (tail is raised and beats about in an unstable manner).
- 2 = Immobile floating. Some vestibular mutant mice that display initial passive immobile floating soon unbalance and display full underwater tumbling or irregular swimming, which should be recorded as such.
- 3 = Underwater tumbling. Mouse is unable to maintain a balanced body position and continuously tumbles. This is an extremely severe behavior and so the mouse must be scooped up and rescued immediately.

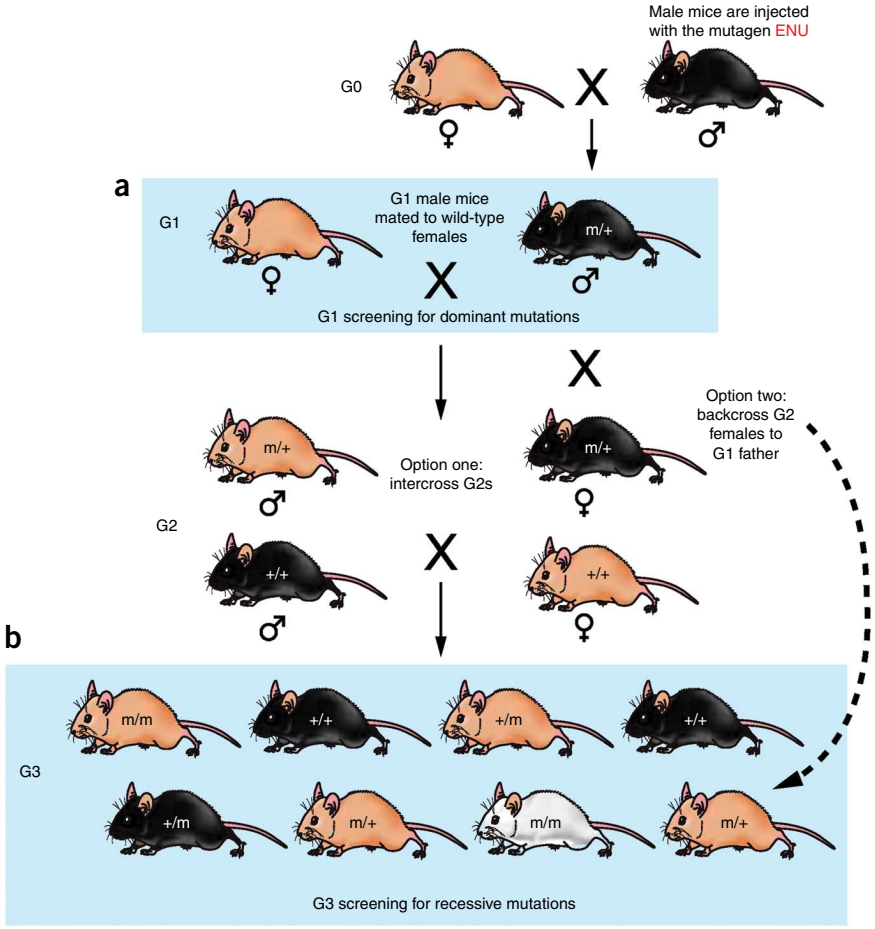

Figure 1 | Breeding schemes for generating both G1 and G3 pedigrees from *N*-ethyl-*N*-nitrosourea (ENU) mutagenized males. (**a**,**b**) G1 (**a**) and G3 (**b**) progeny enter the phenotyping pipelines. In the current mutagenesis pipeline used at MRC Harwell for hearing and vestibular screens, ENU mutagenized C57BL/6 animals are crossed to C3H/HeH to produce G1 progeny for the dominant screen. To generate G3 animals and screen for recessive phenotypes, G2 females are generated and backcrossed back to the founder G1 male. m/+ Mice are heterozygotes carrying an ENU-induced mutation (m), whereas m/m mice are homozygotes. (Adapted and reprinted, with permission, from the *Annual Review of Genomics and Human Genetics*, Volume 9 2008 by Annual Reviews, ref. 4). All animal experiments are carried out according to UK Home Office Guidelines under appropriate licenses.

Tests for auditory function—click-box: The click-box is a handheld unit, which emits a high-frequency (~20 kHz) tone stimulus at a level of 90 dB sound pressure level (dB SPL) when positioned 30 cm above the animal. The click-box will elicit a 'Preyer' reflex in a normal mouse in which the pinnae (outer ear) flick backwards. If the animal being tested exhibits a lack of Preyer reflex or a reduction in comparison to littermates, it is the first indication that the hearing ability is compromised in some way[21]. The advantage of the click-box test is that it is very rapid and enables the efficient testing of large numbers of mutant mice. However, the click-box tests the response to a supra-threshold stimulus and is, therefore, a relatively crude assessment of hearing loss. It should be noted that the Preyer response is often, but not always, accompanied by a startle response whereby the mouse will flinch or jump back to a varying extent. If it is in excessive, then this startle response can also be an

indication of other behavioral phenotypes such as anxiety or stress. As the click-box test is relatively crude, to further categorize and quantify any hearing loss detected by click-box, animals should undergo auditory brainstem response (ABR) testing[22]. Moreover, it is possible to apply ABR testing as a primary testing protocol for all the mice from an ENU screen, though this requires significantly more time and resources than the click-box test.

Tests for auditory function—ABR: The ABR test measures the electrical response in the auditory nerve (CN VIII), and the brainstem, to a defined frequency auditory stimulus (**Fig. 4**). The electrical response detected is amplified and sent via bespoke hardware to a computer that utilizes software to average the response signal and produce a trace that consists of at least five peaks in a normal hearing murine subject (**Fig. 4b**). The main five peaks of the ABR (PI–PV) are thought to have the following origins: PI, auditory nerve; PII, cochlear nucleus; PIII, superior olivary complex; PIV, the vicinity of the preolivary and lateral lemniscal nuclei; and PV contralateral inferior colliculus[23–25]. For each frequency tested, the stimulus level is reduced in 10 dB increments, from a starting level of 90 dB SPL, until no replicable peaks can be observed. The level is then increased by 5 dB to give a final estimation of auditory threshold for the given frequency to within 5 dB SPL (we have adapted this protocol from the website: http://empress.har.mrc.ac.uk (ref. 26) see ref. 15). An audiogram is generated (**Fig. 4d**) that displays the auditory thresholds at different frequencies for both mutant and control wild-type mice and provides a visual guide to the hearing loss in the mutant mouse. There are a number of different ABR systems available, although most will need modification in order to be used with mice. The majority of stand-alone clinical systems do not have transducers capable of delivering the higher stimulus frequencies that are required for assessing auditory function in mice. The equipment we use at MRC Harwell is TDT system III, hardware and software (Tucker Davis Technology (TDT), Alachua, FL, USA) (see **Fig. 4a**). The software enables a large degree of experimental control, and the free-field stimulus transducer, ES1 (TDT), has an almost flat response at frequencies between 4–100 kHz. The transducer is calibrated using a ¼″ measuring microphone (7016 ACO-Pacific, Belmont, CA, USA) and SigCal (TDT) software. Calibration is achieved by measuring the output of the stimulus transducer across the range of ABR test frequencies (8–26 kHz) and creating a normalization file to correct the transducer output by boosting or attenuating the frequencies as required. Once calibration of the speaker has been completed, the stimuli for the ABR test needs to be created by using the SigGen (TDT) software. Tone burst stimuli are gated at the start and end, and total 7 m in length. Separate stimuli or 'signal' files are created using the frequency generation method for each of the test frequencies 8, 12, 20 and 26 kHz, a level variable should be created and values ranging from 90 dB SPL down to 5 dB SPL in 5 dB steps should be entered using the value list method. The normalization file created in SigCal (TDT) should be selected as the calibration method for the signal file. After creating the signal files, a configuration file is created using the BioSig (TDT) software. This file contains the necessary information on stimulus presentation and acquisition of the ABR signal, a configuration file should be created for each of the test

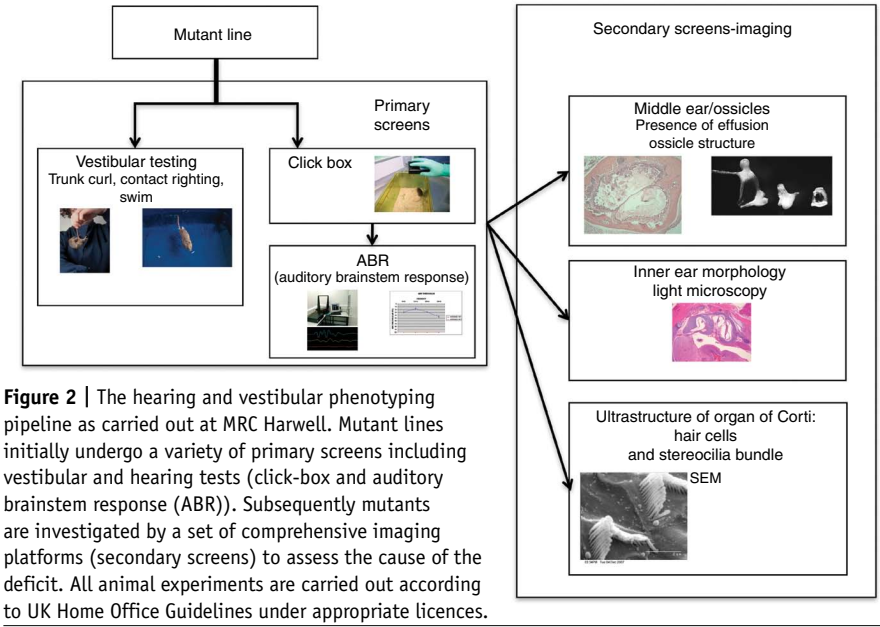

Figure 2 | The hearing and vestibular phenotyping pipeline as carried out at MRC Harwell. Mutant lines initially undergo a variety of primary screens including vestibular and hearing tests (click-box and auditory brainstem response (ABR)). Subsequently mutants are investigated by a set of comprehensive imaging platforms (secondary screens) to assess the cause of the deficit. All animal experiments are carried out according to UK Home Office Guidelines under appropriate licences.

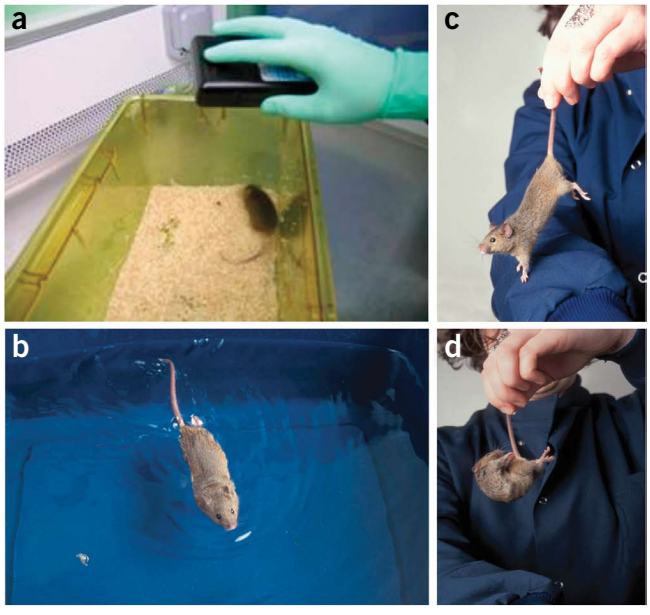

Figure 3 | Primary hearing and vestibular testing of mice. (**a**) Click-box testing. The handheld unit is held 30 cm above the subject and the button depressed (as shown). Mice will respond with a full pinnae flick if there is no hearing impairment. (**b**) A mouse exhibiting normal swimming behavior. The tail is being used to propel the mouse through the water; all four limbs are in motion and the head and back are above the surface of the water. Mice with vestibular anomalies display deficits in these behaviors. (**c**) A normal mouse exhibiting the reaching (trunk curl) response. Mice will reach towards a horizontal landing surface when suspended by their tail. (**d**) In contrast, **d** shows a mouse with a severe vestibular anomaly, curling towards the base of their tail, when suspended. All animal experiments are carried out according to UK Home Office Guidelines under appropriate licences.

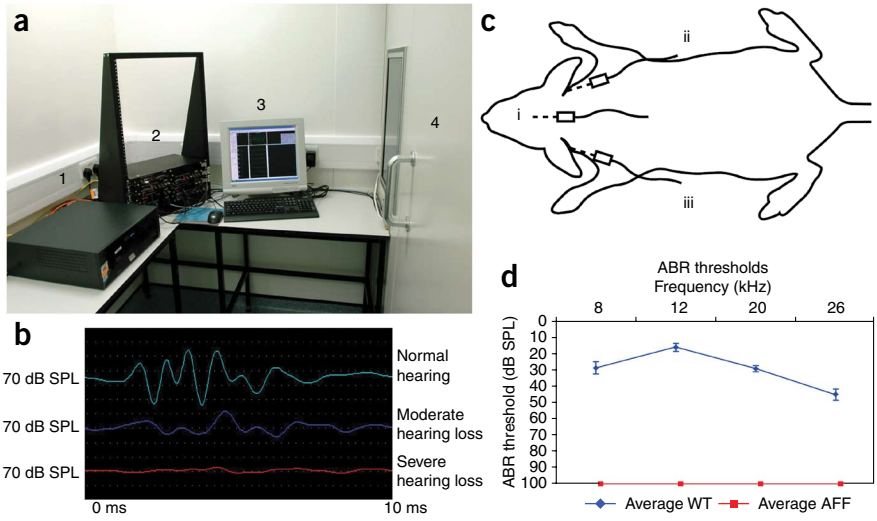

Figure 4 | Auditory brainstem response (ABR) testing. (**a**) The ABR apparatus. The computer (1) is connected to the Tucker Davis Technology (TDT) system III hardware (2), this controls the stimulus speaker in the sound attenuated booth (4), where the mouse is placed on a heated mat for testing. The resulting trace is visualized on the display (3). (**b**) Typical response traces to a 12 kHz tone stimulus at 70 dB sound pressure level (SPL); the light blue trace is from a non-hearing impaired wild-type mouse, the dark blue trace is from a mouse with a moderate hearing loss and the red trace is from a mouse with a severe hearing loss (i.e., no response at 70 dB SPL) (A.P, unpublished data). (**c**) The placement of the sub-dermal electrodes, (i) active, (ii) reference and (iii) ground, the stimulus is delivered free field to the right ear of the subject. The data from the ABR test are presented as an audiogram (**d**), which summarizes the mean hearing thresholds determined at each frequency, with error bars showing the standard error of the mean (in this case $n = 5$) (A.P., unpublished data). No response at the highest stimulus level (90 dB SPL) is recorded as 100 dB SPL for graphical representation. All animal experiments are carried out according to UK Home Office Guidelines under appropriate licences.

frequencies. The file should be set to present the desired stimulus at the rate of 21 s^{-1}, and the response signal should be averaged for 312 repetitions, for the ABR response peaks to become distinguishable from the background signal.

On completion of this battery of auditory and vestibular tests, researchers are left with a comprehensive picture of the peripheral hearing profile and any vestibular effects of the mutation in question. The precise analysis of peaks in an ABR readout will give an indication as to the site of the primary lesion, and the degree of vestibular dysfunction may give an indication of where the aberration may lie. The next stage of the procedure is to carry out imaging analyses to examine the morphology of the ear and to pinpoint the ear pathology that is causing the hearing loss.

Secondary screening of mutant mice. After the identification of mice with a vestibular or auditory deficit, it is important to undertake a variety of investigations to identify the nature of the underlying lesion in the auditory or vestibular system. There are two parts of the ear which merit detailed dissection and structural investigation (as lesions in either or both could be responsible for the observed hearing loss): the middle ear and the inner ear. The middle ear transmits sound from the tympanic membrane via the ossicles to the inner ear. The inner ear comprises the cochlea and the vestibular system, the former being responsible for the process of auditory transduction to the brain.

Examination of the middle ear and the ossicles: Initially, it is important to assess the state of the middle ear. We have observed that the presence of an effusion behind the eardrum, either serous or suppurative may be an indication of otitis media[27–30] (glue ear). Dissection and examination of the ossicles may reveal abnormal development of the ossicular chain from the branchial arches[31] (see **Fig. 5**).

Examination of the inner ear: Subsequently, it is important to undertake dissections (**Fig. 6**) and routine histological analysis of the inner ear. Organs within the inner ear such as the

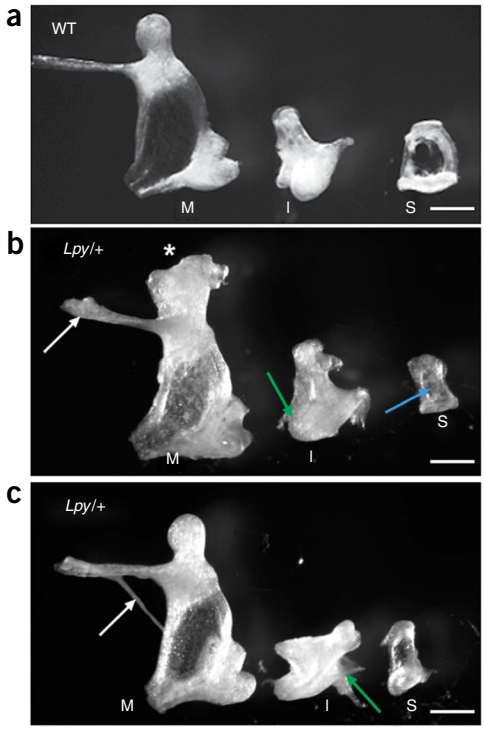

Figure 5 | Dissections of mouse ossicles. (**a**) Three normal ossicles. (**b**,**c**) Two morphologically abnormal sets of ossicles from the dominant deaf mutant, Loopy (*Lpy*) (R.E.H.-H., unpublished data). The green arrows show an abnormal malleal-incudo joint (**b**) and an abnormal short process of the incus (**c**). The blue arrow shows the absence of a hole between the two crura of the stapes. White arrows show abnormalities of the malleus (a bifurcated manubrium (**b**) and an extra 'string' of bone linking the manubrium to the body of the malleus (**c**)). The head of the malleus is also abnormally formed in **b** (*). M, malleus; I, incus; and S, stapes. Scale bars = 100 µm. All animal experiments are carried out according to UK Home Office Guidelines under appropriate licences.

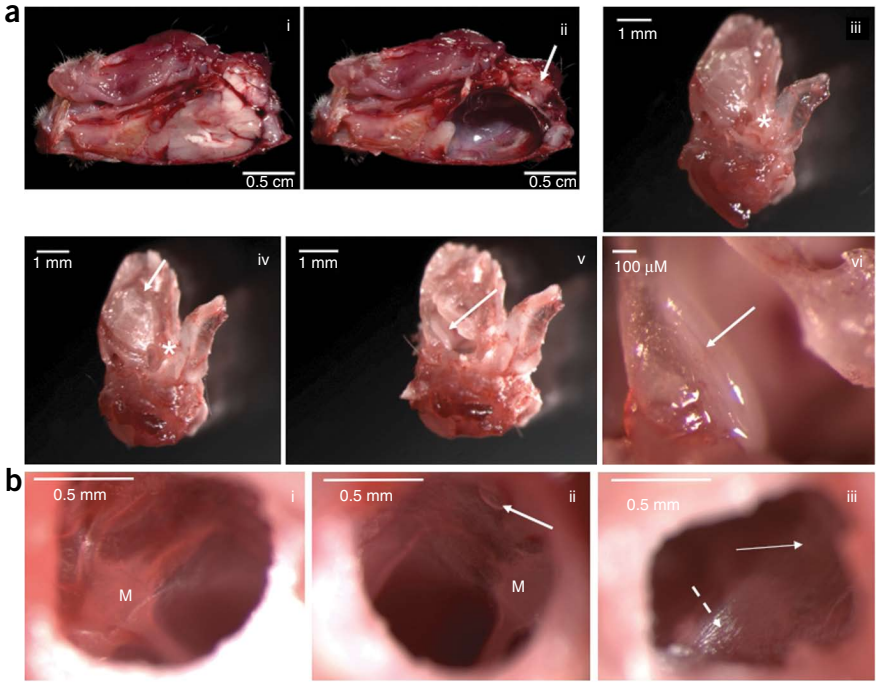

Figure 6 | Dissection of the inner ear for scanning electron microscopy (SEM). (**a**(i)) Half a head (cut side-up) bisected down the midline. After removal of the brain (ii), the inner ear can be clearly seen (white arrow). (iii) After separation from the skull, the inner ear can be seen clearly. The stapes is still attached to the oval window (*). (iv) The stapes is removed (*), and a hole is made in the apex of the cochlea (white arrow). (v) After decalcification the bone is removed to reveal the cochlear duct underneath (white arrow). (vi) The stria is then picked away (white arrow) to reveal the hair cells underneath. (**b**(i)) A wild-type tympanic membrane; the malleus (M) can be clearly seen. (**b**(ii)) A case of otitis media with effusion (OME); note the bubble behind the tympanic membrane (white arrow). (**b**(iii)) A case of supparative otitis media; the tympanic membrane is retracted (dashed white arrow) and pus can be seen behind the tympanic membrane (white arrow). All animal experiments are carried out according to UK Home Office Guidelines under appropriate licences.

endolymphatic compartments can be reconstructed from H&E stained sections using AMIRA software, providing information on structural defects in the inner ear. Observation of the structure of the stria vascularis and the position of Reissner's membrane can indicate defects in endolymph homeostasis[32]. Light microscopy can also be used to observe the structure of otoconia and ampullae in the vestibular system; e.g., missing otoliths can be readily observed (**Fig. 7**).

Ultrastructural studies of the inner ear: Scanning electron microscopy (SEM) is a valuable tool for high-resolution imaging of the structure of the organ of Corti within the cochlea. The organ of Corti contains hair cells that convert vibrations of the basilar membrane into electrical signals that travel along the auditory (VIII) nerve[2]. The hair cells are so called because they project a remarkable array of actin-filled stereocilia from their apical surface. There is one row of inner hair cells (IHCs) and three to four rows of OHCs in a normal healthy organ of Corti. SEM will identify ultrastructural anomalies of the hair cells, including the structure of the stereocilia bundle[33,34] and the pattern and distribution of inner and OHC rows[17] (see e.g., **Fig. 8** and refs. 15,17). SEM utilizes an electron beam that interacts with the gold and palladium atoms on the surface of the specimen. Detectors pick up the interactions to give a signal that can be visualized.

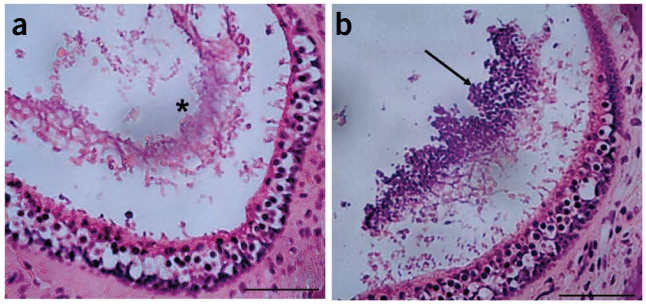

Figure 7 | Parasaggital hematoxylin and eosin (H&E) sections through the saccular maculae of an otoconial mutant mouse and a wild-type mouse. (**a**,**b**) The morphology of a mutant mouse (**a**) and the morphology of a wild-type mouse (**b**) (R.E.H.-H., unpublished data). Note the absence of otoliths in the mutant (*), indicated by an arrow in **b** (wild type). Scale bars = 500 µM.

This protocol describes a phenotyping platform that can be applied in a hierarchical way to the characterization of large numbers of mutants from an ENU screen. First, the simple vestibular and click-box tests, along with ABR testing, can be applied as a primary screen to potentially thousands of mutant progeny (**Fig. 2**). As indicated in the procedure (see below) ABR takes about 35 min to perform and has traditionally not been regarded as a test for primary characterization of mice from mutagenesis pipelines. However, the test does not take much longer than for example complex motor or behavioral tests (e.g., rotarod or open field) that routinely are used for first-line characterization of mutant mice. One operator can analyse upto 10 mice per day, enabling it to be used as a primary phenotyping test.

Overall, these initial tests provide a first-line determination of auditory and vestibular deficits.

Subsequently, we describe a variety of imaging studies that can be applied as a secondary screen that will aid in the determination of the underlying pathology. However, the tests described are those that we routinely apply for initial characterization of deaf mutants following their identification in the primary deafness screen. They are not comprehensive in that other relatively sophisticated tests can be applied at the secondary stage to further elaborate the nature of the auditory deficit. These include DPOAE, to examine OHC function[15]; otoscopy[35], to image effusion in the middle ear in live animals; and tympanometry, to assess movement of the tympanic membrane, which may be impaired in conductive hearing losses[36].

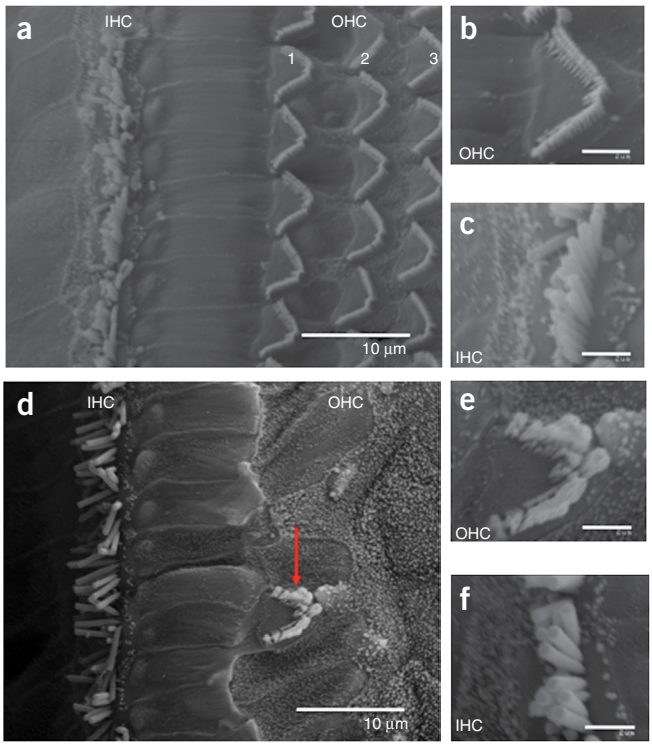

Figure 8 | Scanning electron micrographs showing the sensory hair cells on the organ of Corti of a wild-type mouse. (**a**) The one row of inner hair cells (IHC) and three rows of outer hair cells (OHC). (**b**) A stereocilia bundle of an OHC (note the W configuration of the stereocilia). (**c**) The stereocilia bundle of one IHC. (**d**) The organ of Corti from a sensorineural mutant (R.E.H.-H., unpublished data). In this patch there is only one OHC visible (red arrow). (**e**) A higher magnification of this stereocilia bundle, even though the three rows are still in evidence there is obvious fusion of the stereocilia. (**f**) An IHC in higher magnification. It should be noted that the bundle comprises fewer stereocilia, and they are thicker and more rod-like (compared with **c**). **a**,**d** scale bars = 10 µm, **b**,**c**,**e**,**f** scale bars = 2 µm. All animal experiments are carried out according to UK Home Office Guidelines under appropriate licences.

MATERIALS

REAGENTS

Enu mutagenesis

- Mice inbred strains: C57BL/6; C3H/HeH **! CAUTION** All experiments using animals should conform to the institutional and national guidelines and be appropriately licensed.
- ENU (Sigma-Aldrich, cat. no. N3385) **! CAUTION** May cause cancer, heritable genetic damage or harm to the unborn child. Also harmful by inhalation, contact with skin and if swallowed. Wear gloves, face mask and protective gown when handling, and handle in a fume hood.

Hearing testing

ABR

- Anesthetics:
- Ketamine (Vetalar V) (Pfizer) **! CAUTION** This is a controlled substance. It is severely irritating to the eyes and has chronic effects on the CNS. Wear gloves when handling. (Avertin may be used as an alternative.)
- Medetomidine (Sedator) (Dechra) **! CAUTION** Harmful if swallowed. Wear gloves when handling.
- Atipamezole (Antisedan) (Pfizer)

Imaging

SEM

- Gluteraldehyde (Sigma-Aldrich, cat. no. G5882) **! CAUTION** Harmful if swallowed, toxic by inhalation, causes burns and may cause sensitization by inhalation and skin contact. Wear laboratory coat and gloves when handling. Handle in a fume hood.
- Sodium phosphate monobasic (Sigma, cat. no. S-3139)
- Sodium phosphate dibasic (Sigma, cat. no. S-3264)
- 4% Osmium tetroxide (wt/vol) (Agar, cat. no. R1024) **! CAUTION** Osmium tetroxide is very toxic by inhalation and causes burns on skin contact and if swallowed. Wear laboratory coat and gloves when handling. Handle in a fume hood.
- Sodium cacodylate buffer (Sigma, cat. no. C0250) **! CAUTION** Sodium cacodylate is toxic by inhalation and if swallowed. Also toxic to aquatic organisms. Wear laboratory coat and gloves when handling. Handle in a fume hood.
- Thiocarbohydrazide (Fluka, cat. no. 88535) **! CAUTION** Very toxic by inhalation and if swallowed. Heating may cause an explosion. Wear laboratory coat and gloves when handling. Handle in a fume hood.
- Ethanol (25, 40, 60, 80, 95 and 100%) (Fisher Scientific, cat. no. E/0650DF/17) **! CAUTION** Flammable. Wear laboratory coat and gloves when handling.
- Liquid (pressurized) CO_2
- Gold palladium alloy (Agar Scientific, cat. no. B7371)

Histology and light microscopy

- 10% (vol/vol) neutral-buffered formaldehyde (Surgipath Europe, cat. no. UN2209) **! CAUTION** Harmful by inhalation, in contact with skin and if swallowed, limited evidence of a carcinogenic effect and may cause sensitization by skin contact.
- D.F.B.—(decalcifiying agent—H.K Kristenson) (Pioneer Research Chemicals, cat. no. PRC/R/12) **! CAUTION** Avoid contact with skin. Wear laboratory coat, gloves and safety glasses when handling. Handle in a fume hood.
- Industrial methylated spirit (Chemix, cat. no. IMS740P) **! CAUTION** Highly flammable. May be harmful by inhalation, ingestion or skin absorption. May act as an irritant. Wear laboratory coat, gloves and safety glasses when handling. Handle in a fume hood.
- Sub-X (xylene substitute clearing agent) (Surgipath Europe, cat. no. 03762E) **! CAUTION** Flammable. May be harmful by inhalation, ingestion or skin absorption. May act as an irritant. Wear laboratory coat, gloves and safety glasses when handling. Handle in a fume hood.
- Paraffin wax (Surgipath Europe, cat. no. EM400) **! CAUTION** Keep away from heat and ignition sources, harmful if swallowed. Avoid inhaling the vapor, use with adequate ventilation. Avoid contact with eyes, skin and clothes. Wear laboratory coat and gloves when handling.
- Hematoxylin (Shandon Gill 3, Thermoscientific, cat. no. 6765009) **! CAUTION** May be harmful if swallowed; eye, skin and respiratory irritant. Wear laboratory coat and gloves when handling.
- Eosin (Shandon Eosin Y (aqueous), Thermo Electron, cat. no. 6766010)
- Hydrochloric acid (Acros Organics, cat. no. 124630026) **! CAUTION** Toxic and corrosive. Keep away from heat and ignition sources, harmful if swallowed. Avoid inhaling the vapor, use with adequate ventilation. Avoid contact with eyes, skin and clothes. Harmful for aquatic organisms. Wear laboratory coat, gloves and safety glasses when handling. Handle in a fume hood.
- Isopropanol (Fisher Scientific, cat. no. P/7490/17) **! CAUTION** Highly flammable. May be harmful by inhalation, ingestion or skin absorption. May act as an irritant. Wear laboratory coat, gloves and safety glasses when handling. Handle in a fume hood.
- Lithium carbonate (Fischer Scientific, cat. no. 19336-1000) **! CAUTION** Harmful if swallowed or inhaled. Wear laboratory coat, gloves and safety glasses when handling. Handle in a fume hood.
- Clearium (Surgipath Europe, cat. no. 090510) **! CAUTION** Highly flammable. May be fatal if swallowed. Harmful by inhalation or skin contact. Wear laboratory coat, gloves and safety glasses when handling. Handle in a fume hood.

EQUIPMENT

Vestibular testing

- Contact righting tube (a transparent perspex tube with a 3 cm circular diameter), ~22 cm in length (manufactured at MRC Harwell)
- Baby bath or similar container that allows the mouse to swim freely in an open space
- A cage top or similar horizontal surface so that the mouse can be held above it for the trunk curl test

Hearing testing
- A click-box—a handheld unit which emits a high frequency (~20 kHz) tone stimulus at a level of 90 dB sound pressure level (dB SPL) when positioned 30 cm above the animal (obtained from the MRC Institute of Hearing Research)
- Heated cage (Datesand)
- Audiometric chamber (IAC, cat. no. 401-A-SE)
- Heated mat
- ES1 Free field transducer (TDT)
- SigGen/BioSig software (TDT)
- TDT system III hardware: RP2.1 processor (X2), ED1 speaker driver, RA4-PA pre-amplifier, RA16 Medusa base station and MA3 microphone amplifier (TDT)
- ¼″ Measuring microphone (ACO-Pacific, cat. no. 7016)
- SigCal software (TDT)
- Electrodes (Grass Telefactor, cat. no. F-E2-12)

Imaging
- Histological sample processing—ThermoShandon Pathcentre (Thermo Scientific)
- Tissue embedding centre (R.A. Lamb)
- Microtome—Finesse ME (ThermoShandon)
- S35 Feather disposable microtome blade (Surgipath Europe, cat. no. 08310E-S35)
- Glass slides (Fischer Scientific, cat. no. MNJ-250-005L)
- Glass coverslips (Fischer Scientific, cat. no. MNJ-350-090J)
- H&E staining—ThermoShandon Varistain 24-4 (Thermo Scientific)
- Critical point drying—Emitech K850 (EM Technologies)
- Sputter coating (Polaron instruments, cat. no. E5100)
- SEM (S-530, Hitachi)
- Dissection microscope with a cold light source (side illumination)
- Standard stereo bright-field slide microscope

REAGENT SETUP

1% Acid alcohol Dilute hydrochloric acid to 1% in 70% methylated spirits. Store in flammable liquid safety cabinet indefinitely.
! CAUTION Hydrochloric acid is toxic and corrosive. Keep away from heat and ignition sources, harmful if swallowed. Avoid inhaling the vapors, use with adequate ventilation. Avoid contact with eyes, skin and clothes. Harmful for aquatic organisms. Wear laboratory coat, gloves and safety glasses when handling. Handle in a fume hood. **! CAUTION** Industrial methylated spirit is highly flammable. May be harmful by inhalation, ingestion or skin absorption. May act as an irritant. Wear laboratory coat, gloves and safety glasses when handling. Handle in a fume hood.

2.5% Glutaraldehyde (wt/vol) Dilute 25% glutaraldehyde solution in 0.1 M phosphate buffer. The solution must be freshly prepared on the day of use. **! CAUTION** Glutaraldehyde is harmful if swallowed, toxic by inhalation, causes burns and may cause sensitization by inhalation and skin contact. Wear laboratory coat, gloves and safety glasses when handling. Handle in a fume hood.

0.1 M Phosphate buffer Add 1.48 g sodium phosphate monobasic and 5.68 g sodium phosphate dibasic to 263 ml of water, mix well until dissolved. Store at 4 °C for up to 1 month.

1% (wt/vol) Osmium tetroxide Dilute 4% (wt/vol) osmium tetroxide in 0.1 M sodium cacodylate buffer to a 1% final concentration. 1% osmium tetroxide (wt/vol) should be made fresh on the day of use and left at room temperature in fume hood until protocol is complete. Unused solution should be disposed of in an appropriate waste container. **! CAUTION** Osmium tetroxide is very toxic by inhalation and causes burns on skin contact and if swallowed. Wear laboratory coat, gloves and safety glasses when handling. Handle in a fume hood. **! CAUTION** Sodium cacodylate is toxic by inhalation and if swallowed. It is also toxic for aquatic organisms. Wear laboratory coat, gloves and safety glasses when handling. Handle in a fume hood.

1% (wt/vol) Thiocarbohydrazide Make a 1% solution in water (1 g of thiocarbohydazide in 100 ml of water) and filter sterilize with a 0.2 µm sized filter. 1% thiocarbohydrazide (wt/vol) should be made fresh on the day of use and left at room temperature in fume hood until protocol is complete. Unused solution should be disposed of in an appropriate waste container. **! CAUTION** Thiocarbohydrazide is very toxic by inhalation and if swallowed. Heating may cause an explosion. Wear laboratory coat, gloves and safety glasses when handling. Handle in a fume hood.

PROCEDURE

Simple primary screening of mutant mice—vestibular testing ● TIMING 8 min

1| Observe the mouse in the home cage and record signs of circling, head bobbing/weaving, head tilting and abnormal gait, termed *shaker–waltzer* behavior and indicative of a vestibular dysfunction.
! CAUTION All experiments using animals should conform to the institutional and national guidelines and must be appropriately licensed.

2| *Trunk curl test*: Hold the mouse by the tail and present the mouse with a horizontal surface. Observe if the mouse reaches out to the horizontal surface or if it curls its trunk towards its tail (**Fig. 3c,d**).

3| *Contact righting test*: Place the mouse in a Perspex tube (3 cm in diameter) so that all its four feet are in contact with the bottom of the tube and the animals back is in contact with the upper side of the tube. Rotate the tube through 180°, turning the animal upside down. Observe if the mouse re-orientates in the tube.

4| *Swim test*: Place either a standard desk lamp above the home cage or a heating mat (set to 37 °C) in the cage; alternatively a heated plate (set to 37 °C) can be placed below the cage for drying the mouse after the swim test.

5| Fill a container (such as a large washing up bowl, large bucket or even a sink) with at least 15 cm depth of water at 24–26 °C, which will allow free movement swimming of the mouse.

6| Remove the mouse from its home cage by gripping the tail between the thumb and forefinger and lowering it carefully into the water.

7| Observe the mouse for a maximum of 1 min and assess its swimming ability (**Fig. 3b**). Continue to observe the mouse for the maximum of 1 min to see if it displays passive immobile floating. Mice should be scooped up and rescued immediately on displaying underwater tumbling or any distressing behavior.

Simple primary screening of mutant mice—use of a click-box ● TIMING 1 min

8| Position a click-box 30 cm above the animal in its home cage and depress the key (**Fig. 3a**)
? TROUBLESHOOTING

9| Observe the presence or absence of a 'Preyer' reflex in which the pinnae of the mouse flick backwards in response to the tone emitted by the click-box.
? TROUBLESHOOTING

Primary auditory phenotyping platforms—ABR ● TIMING 35 min, with an additional 3 h if the procedure requires recovery
10| Prepare the anesthetic and muscle relaxant by combining 0.38 ml of 1 mg ml^{-1} ketamine (Vetalar V) and 0.5 ml of 100 mg ml^{-1} medetomidine (Sedator) with 4.12 ml of sterile water. Anesthetize a mouse by intraperitoneal injection of the mixture at a dose of 0.1 ml 10 g^{-1} of body weight, place the mouse in a heated cage (~37 °C) to maintain its body temperature and observe until movement has ceased.
! CAUTION Ketamine is a controlled substance, is severely irritating for the eyes and has chronic effects on the CNS. Avertin may be used as an alternative.
! CAUTION Medetomidine is harmful if swallowed.

11| Place the mouse in an audiometric chamber on a heated mat (~37 °C), with the right ear positioned 1.5 cm away from the center of the free field transducer.

12| Place three sub-dermal recording electrodes over the vertex (active), right mastoid (reference) and left mastoid (ground) (see **Fig. 4c**), check on RP2.1 processor that the clip light is not flashing.
? TROUBLESHOOTING

13| Load the 8 kHz configuration file created, as described in the Experimental design section, into the BioSig software and click the 'Start' tab on the left hand side of the screen to commence stimulus delivery. This action will prompt to create an appropriate file name and path for the response file. Mouse identification and additional information may be entered at this point by clicking the 'Subject Info' tab in the setup menu. Ensure that the RP filter section is set to high pass at 300 Hz and low pass at 3 kHz to remove unnecessary noise from the signal. The ABR trace should be observable in the running average window; check that this signal has clearly visible peaks before commencing acquisition.
? TROUBLESHOOTING

14| Click the 'Modify Schedule' tab in the S.G.I. control section of the BioSig software display, select 90–40 dB SPL in 10 dB steps, and 35–5 dB SPL in 5 dB steps from the variable list. Commence acquisition by clicking the 'Begin' tab.

15| Observe the ABR trace in the running average window. After each set of averages, the resulting ABR trace will be placed in the history window and may be dragged to the worksheet for comparison (**Fig. 4b**). When the ABR trace in the running average window begins to lose definition, select the 'Each Thrice' box to repeat the stimulus level three times. Estimation of the ABR threshold is defined as the stimulus level for the given frequency, that the last replicable peak (that can be identified as one of the first five ABR peaks) is visible. A 1500 Hz low-pass filter may be selected from the 'calculator' menu and applied to the response traces in the worksheet to further reduce noise, thus enabling more accurate threshold estimation at low-response trace amplitudes.
? TROUBLESHOOTING

16| If the ABR threshold is above 40 dB SPL, a stimulus of 5 dB SPL above this level should be applied by selecting in the 'Modify Schedule' menu of the S.G.I. control, to give an estimation of the threshold to within 5 dB SPL. Repeat Steps 13–16 using the 12, 20 and 26 kHz BioSig configuration files.
? TROUBLESHOOTING

17| If recovery is required, administer atipamezole (Antisedan, 5 mg ml^{-1}) subcutaneously at a dose of 1 mg kg^{-1} of body weight to aid the acceleration of recovery. The mouse must be returned to the heated cage (~37 °C) until normal movement is observed, and must subsequently be moved to its home cage and monitored until complete recovery.

Secondary screens—imaging the ear
18| The imaging of the ear in secondary screens can be carried out using option A, visual examination of the middle ear and the ossicles; option B, examination of the inner ear using light microscopy; or option C, ultrastructural studies of the inner ear using SEM.

(A) Examination of the middle ear and the ossicles ● TIMING 35 min

(i) Kill the mouse using a protocol recommended by the local guidelines.

(ii) Remove the external ears (the pinnae) and position the head so that the tympanic membrane is visible through a dissecting microscope.

(iii) Score the ears for the presence of fluid effusion behind the eardrum (if this is a serous effusion, there are often bubbles in the fluid that can readily be visualized). (**Fig. 6bii**). Also examine the ears for the presence of a suppurative effusion (pus) (**Fig. 6biii**) and tympanosclerosis (calcium deposits on the eardrum, the white deposits are easily detectable) or any perforations in the eardrum.

(iv) Perforate the eardrum with watchmakers forceps and carefully separate and remove the ossicles. Observe the ossicle morphology and compare to a wild-type mouse (e.g., see **Fig. 5**).

(B) Examination of the inner ear ● TIMING 2 weeks

(i) Kill the mouse by cervical dislocation.

(ii) Remove the head and skin, and bisect down the midline. Fix the two halves of the head by submersion in 15 ml of 10% (vol/vol) neutral-buffered formaldehyde, for 24 h at room temperature (20 °C).
! CAUTION Neutral-buffered formaldehyde is harmful by inhalation, in contact with skin and if swallowed, has limited evidence of a carcinogenic effect, and may cause sensitization by skin contact.

(iii) Remove the two halves of the head from the fixative and de-calcify the fixed specimens by submersion in 50 ml of D.F.B., for 2 d at room temperature in a fume hood.

(iv) Remove the samples from the decalcifying solution, rinse with water and place in ThermoShandon Pathcentre for automated dehydration by submersion in graded industrial methylated spirit (70, 90 and 100%), clearing with Sub-X and coating in paraffin wax.

(v) Transfer the sample from the ThermoShandon Pathcentre into the tissue-embedding center to embed in a fresh paraffin wax block and leave to solidify.
! CAUTION Keep paraffin wax away from heat and ignition sources; harmful if swallowed. Avoid inhaling the vapors; use with adequate ventilation. Avoid contact with eyes, skin and clothes. Wear laboratory coat and gloves when handling.

(vi) Place the paraffin wax block in the chuck of a microtome and trim around the sample at a section thickness of 15 μm leaving a prism shape.
▲ CRITICAL STEP Cutting of sections for histology is a highly skilled procedure and should not be attempted without appropriate training from an experienced histologist.

(vii) Float sections onto clean, warm distilled water (50 °C) to remove creases and place on the glass slides.

(viii) Perform hematoxylin and eosin (H&E) staining using the Shandon Varistain 24 to automate solution changes; remove the paraffin from the slide by submersion in Sub-X, before rehydration is achieved by graded submersion isopropanol (70, 100%) and running water. Immerse the slide in hematoxylin for 6 min to stain the basophilic structures such as the nucleus blue or purple, rinsed in running water before immersing in 1% (vol/vol) acid alcohol to remove the background. Rinse the slide in running water, and immerse in 1% (wt/vol) lithium carbonate to 'blue' the nuclei of the cells. Rinse the slide again in water before submersion in eosin for 3 min to counterstain the eosinophilic structures such as the cytoplasm pink. Rinse the slide in running water once more before dehydration is achieved by several changes of isopropanol.
! CAUTION Sub-X is flammable. May be harmful by inhalation, ingestion or skin absorption. May act as an irritant. Wear laboratory coat, gloves and safety glasses when handling. Handle in a fume hood.
! CAUTION Isopropanol is highly flammable. May be harmful by inhalation, ingestion or skin absorption. May act as an irritant. Wear laboratory coat, gloves and safety glasses when handling. Handle in a fume hood.
! CAUTION Hematoxylin may be harmful if swallowed; eye, skin and respiratory irritant. Wear laboratory coat and gloves when handling.
! CAUTION Lithium carbonate is harmful if swallowed or inhaled. Wear laboratory coat, gloves and safety glasses when handling. Handle in a fume hood.

(ix) Transfer the slide to a fume hood in a pot containing isopropanol and coverslip in fume hood using Clearium as a mounting solution.
! CAUTION Clearium is highly flammable. May be fatal if swallowed. Harmful by inhalation or skin contact. Wear laboratory coat, gloves and safety glasses when handling. Handle in a fume hood.

(x) Examine the H&E stained slides by bright-field light microscopy. Observe if all the structures of the inner ear are present, in the correct orientation and are of the correct size with the expected number of cells. Observe the cochlear duct to check that the appropriate number of rows of hair cells are present, the membranes are in the correct place, the cochlea is of normal length and the layers of the stria vascularis look normal. In the vestibular system, observe to verify whether all the sensory patches are present (the three ampullae of the semi-circular canals and the two gravity receptors (the utriculus and the sacculus). Also note the presence or absence of the semi-circular canals. If the inner

ear appears normal with light microscopy, then the defect may be more subtle and may only be detectable using SEM (ultrastructural studies).

(C) Ultrastructural studies of the inner ear—SEM ● TIMING 5 d

(i) Kill the mouse by cervical dislocation.

(ii) Remove the head, skin and bisect down the midline.

(iii) Bisect the head and remove the brain. Identify the position of the inner ear, which is identified by the superior semi-circular canal (**Fig. 6ai,ii**).

▲ CRITICAL STEP The dissection must be carried out efficiently (in under 2 min) to minimize fixation artifacts.

(iv) Remove the extraneous tissue from around the ear, leaving the inner ear and surrounding the middle ear bulla.

(v) Gently lift the bulla away from the inner ear by the gentle insertion of one point of a pair of watchmaker's forceps into the region of the ear canal where the tympanic membrane resides.

(vi) Discard the bulla and remove the stapes by placing one arm of a pair of watchmaker's forceps in the gap between the two arms and pulling (**Fig. 6a,iii**). This serves to 'open up' the fluid space in the inner ear.

(vii) Make another hole in the apex of the cochlea by gently 'scratching' the bony shell away (**Fig. 6a,iv**). This will allow the fixative exchange when the specimen is being fixed.

(viii) Place the inner ears in 5 ml of 2.5% gluteraldehyde (wt/vol) in a 0.1 M potassium phosphate buffer solution and fix overnight at 4 °C.

! CAUTION Glutaraldehyde is harmful if swallowed, toxic by inhalation, causes burns and may cause sensitization by inhalation and skin contact.

(ix) Following fixation and decalcification, snip the bone between the oval windows and pick away the bony shell of the inner ear revealing the cochlear duct (**Fig. 6a,v**).

(x) Identify the stria vascularis by the dark pigmentation of the melanocytes (**Fig. 6a,vi**). Carefully remove this using fine (size 5) watchmaker's forceps, revealing the hair cell rows underneath.

(xi) Place the dissected inner ear in 1% (wt/vol) osmium tetroxide (in 0.1 M sodium cacodylate) at room temperature (20 °C) for 1 h in a glass container.

! CAUTION Osmium Tetroxide is very toxic by inhalation, causes burns on skin contact and if swallowed.

! CAUTION Sodium cacodylate is toxic by inhalation and if swallowed. Also toxic to aquatic organisms.

(xii) Wash the samples in water six times, each for 3 min at room temperature.

(xiii) Place the specimens in 1% thiocarbohydrazide (wt/vol) for 30 min at room temperature.

! CAUTION Thiocarbohydrazide is very toxic by inhalation and if swallowed. Heating may cause an explosion.

(xiv) Repeat Steps (xi)–(xiii).

(xv) Repeat Steps (xi)–(xiii).

(xvi) Repeat Steps (xi)–(xii).

(xvii) Submerse the specimens in increasing strength ethanol solutions for dehydration (25, 40, 60, 85 and 95%, each for 45 min at 4 °C).

(xviii) Incubate the specimens in 100% ethanol for 30 min at 4 °C.

(xix) Repeat Step (xviii) twice.

■ PAUSE POINT Samples can be stored in 100% ethanol for several weeks at room temperature.

(xx) Remove the specimen from ethanol and place in the critical point dryer. Once all the moisture has been removed from the specimen (this is indicated by placing a piece of filter paper in front of the emission pipe at the back of the critical point dryer and it remaining dry), apply an ultra thin layer of an electrically conductive material such as gold–palladium alloy using a sputter coater.

(xxi) Place the specimen in the chamber of an SEM system and scan.

(xxii) Capture and store images from different areas of the cochlear turns, including basal, middle and apical turns.

? TROUBLESHOOTING

● TIMING

Steps 1–7, Simple primary screening of mutant mice: vestibular testing: 8 min per mouse

Steps 8–17, Auditory phenotyping—Click-box and ABR: 35 min per mouse (+3 h if recovery is required)

Step 18A, Secondary screens—examination of the middle ear and the ossicles: 35 min per mouse

Step 18B, Secondary screens—examination of the inner ear: 2 weeks per mouse

Step 18C, Ultrastructural studies of the inner ear—SEM: 5 d per mouse

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1.**

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
Hearing tests			
8–9	No response in wild-type mice	Flat battery or noise not loud enough	Replace batteries and recalibrate instrument with appropriate sound analysis equipment
12	Clipping light comes on	Electrodes are not correctly positioned	Reposition electrodes (see Step 12)
13	No recognizable peaks in a wild-type mouse	Electrodes are not correctly positioned Connections are loose	Reposition electrodes (see Step 12) Check connection ports
15 and 16	Noisy trace-masking of peaks	Mispositioned electrodes, insufficient anesthetic levels, movement of animals	Reposition electrodes (see Step 12) Check connection ports and position of the animal
Scanning electron microscopy (SEM)			
18C (xxii)	Specimen blebbing	Fixation not rapid enough (see 18(iii))	Repeat experiment
	Flaring of electrons on specimen	Insufficient gold thickness	Remove specimen from SEM and re-coat (Step 18C (xx))

ANTICIPATED RESULTS

The outcome of this protocol is the identification and characterization of new mouse models of genetic deafness.

Pardon (*Emx2*), a mutant showing a middle ear ossicle defect

The dominant mouse mutant, Pardon, was identified from the Harwell ENU mutagenesis screen[21] as it showed an absent Preyer reflex in response to the click-box test[31]. Further investigation of this mutant demonstrated both middle ear and cochlear defects (see **Fig. 9**). Dissection of the middle ear showed that all the ossicles—the malleus, the incus and the stapes—were malformed and presumably accounted for some part of the hearing loss. Examination of the organ of Corti demonstrated that in the apical and middle turns of the cochlea there was a patterning defect manifested by additional rows of OHCs, accounting for a significant increase in OHC numbers in this region of the cochlea. In addition, we found that there was disorganization of IHCs, with some IHCs appearing to form a second row (**Fig. 9**). The disorganization of hair cells was reflected in measurements of compound action potentials from the inner ear, which had significantly elevated thresholds in *Pardon* mutants. Pardon was shown to carry a missense mutation in the homeobox gene, *Emx2* (ref. 31).

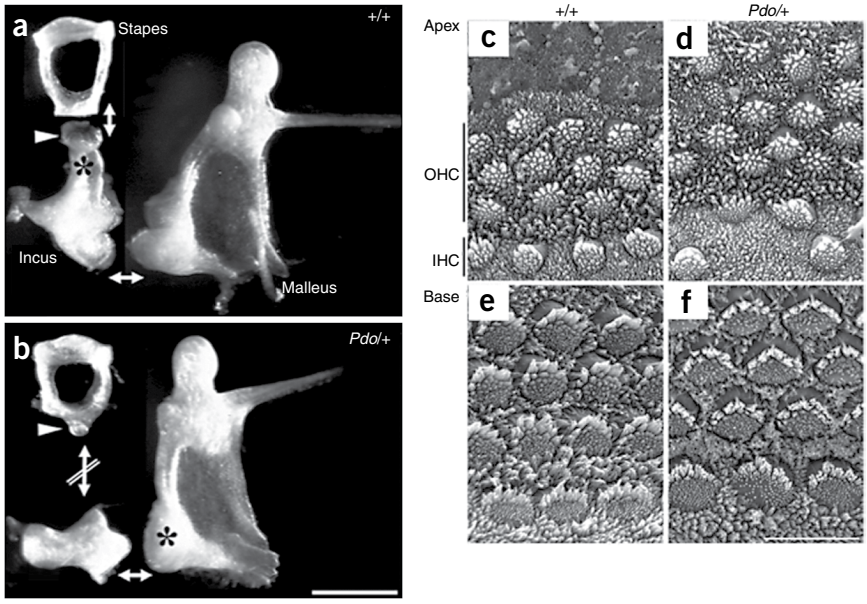

Figure 9 | Middle ear ossicles from P20 *Pdo/+* mutants (**a**) and controls (**b**) showing malformed articulatory surfaces in all three bones. Malleus in P20 *Pdo/+* mice has a malformed articulatory surface (asterisk) (**a**) compared with wild type (**b**). The incus in *Pdo/+* mice lacks the long process (asterisk in +/+) and the lenticular process (arrow head in +/+). The head of the stapes displays a 'bobble' shape (arrowhead in *Pdo/+*) instead of the flat surface in the control. Scale bar = 500 µm. (**c**–**f**) Scanning electron micrographs of the organ of Corti in newborn *Pdo/+* mice demonstrate neuroepithelial defects. Views of the apical coil in the control (+/+, **c**) and mutant (*Pdo/+*, **d**), showing four rows of outer hair cells (OHC) and a more irregular organization of the inner hair cells (IHC) in the mutant. The basal coil in the control (**e**) and the mutant (**f**) show a more regular arrangement in the mutant. Scale bar = 10 µm. Adapted and reprinted, with permission from the Association for Research in Otolaryngology, reference 31.

Jeff (*Fbxo11*) and *Junbo* (*Evi1*), mutants showing middle ear inflammatory disease (otitis media)

As part of the Harwell ENU mutagenesis program[21], two dominant mutants—*Jeff* and *Junbo*—were identified by lack of a robust Preyer reflex in response to the click box[29,30]. Neither mutant showed any vestibular abnormality. Middle ear dissections demonstrated no defects in the ossicular chain. However, examination of the middle ears uncovered an effusion in both mutants (**Fig. 10**) presumably leading to a conductive hearing loss that accounted, at least, in part, for the poor response to the click-box. Histology of the inner ear failed to identify any abnormality in the organ of Corti. However, histology of the middle ear in both mutants revealed a typical chronic suppurative otitis media with a granulocytic effusion and a thickened mucoperisoteum and edematous polyps projecting into the middle ear cavity.

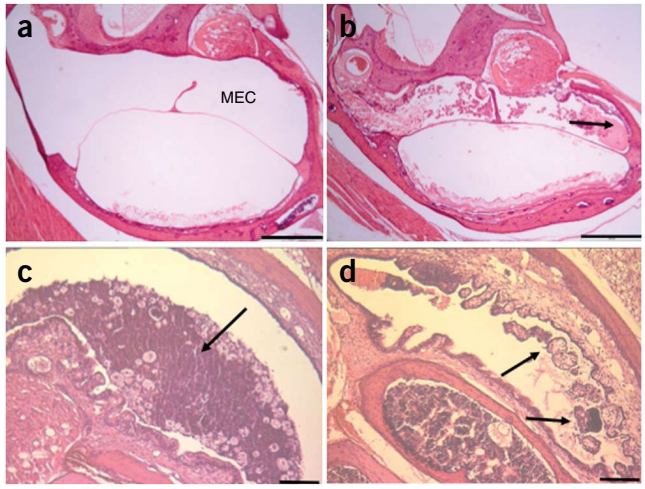

Figure 10 | Histopathology of the middle ear. Wild-type (**a**) and *Jf*/+ mice (**b**–**d**), showing middle ear glue (**b**, **c**, arrows) and polypoid exophytic growths that project into the middle ear cavity (**d**, arrows). Adapted and reprinted, with permission, from reference 29.

Both of these mutants, thus, display a similar pathology that models the chronic disease pathology seen in humans. However, each mutant maps to a different locus on the mouse genome. *Jeff* has been shown to carry a missense mutation in the F-box gene, *Fbxo11* (ref. 27). *Junbo* carries a mutation in the transcription factor, *Evi1* (ref. 30). The identification of these two novel models of otitis media and the discovery of the underlying genes contributes to our understanding of the genetic pathways involved with chronic otitis media in the human population.

Cloth-ears (*Scn8a*^{Clth}), a mutant showing a defect in higher auditory peripheral function

Cloth-ears (*Scn8a*^{Clth})[15] was identified from the Harwell ENU mutagenesis program[21]. Cloth-ears heterozygotes (*Scn8a*^{Clth}/+) showed a mild or absent Preyer reflex response to the click-box. *Scn8a*^{Clth}/*Scn8a*^{Clth} homozygotes also displayed a reduced startle response to the click-box. However, vestibular testing showed no deficit in the vestibular system. Subsequently, we examined the hearing loss in Cloth-ears mice using ABR (**Fig. 11**). Auditory thresholds of *Scn8a*^{Clth}/+ mice were significantly increased compared with +/+ mice at 8 kHz. *Scn8a*^{Clth}/*Scn8a*^{Clth} thresholds were also significantly increased compared with +/+ mice across all four frequencies tested, thus indicating that Cloth-ears has a mild semi-dominant hearing loss. To investigate the pathobiology of the hearing loss in Cloth-ears mice, we undertook a variety of secondary screens that are integral to our pipeline. Dissection of the middle ear revealed no evidence of an otitis media. Furthermore, dissection and X-ray analysis of the middle ear ossicles showed no malformations compared with the wild-type controls. We proceeded in the phenotype pipeline to investigate the ultrastructure of the inner ear analyzing H&E sections of the cochlea and carrying out SEM analysis of the organ of Corti. However, H&E sections were normal and SEM analysis of cochlear hair cells did not reveal any stereocilia abnormalities or hair cell degeneration.

Overall, the data indicated a defect in higher peripheral neural auditory regions and we returned to our ABR analysis and examined the detailed ABR waveforms in Cloth-ears, as the different peaks correspond to electrical signals generated by

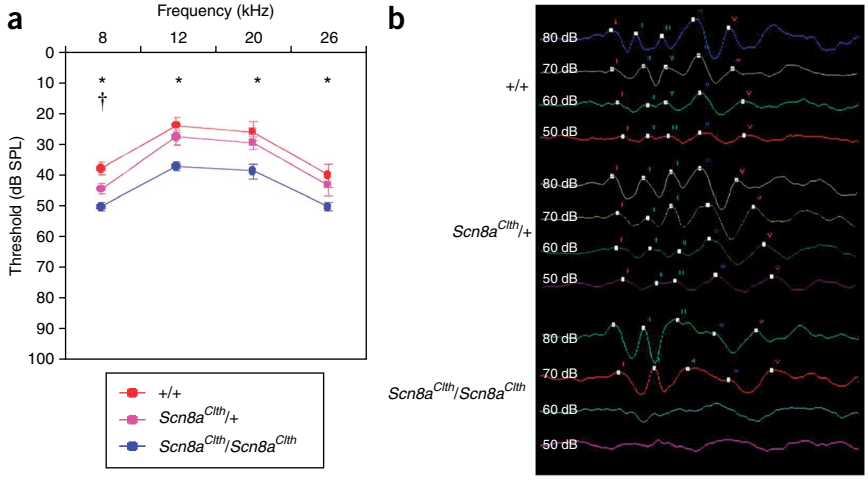

Figure 11 | Auditory brainstem response (ABR) analysis of the Cloth-ears mutant. (**a**) An audiogram plotting the average ABR thresholds and standard error of the mean of 3-month-old sex-matched +/+ (n = 7), *Scn8a*^{Clth} / +(n = 7) and *Scn8a*^{Clth} / *Scn8a*^{Clth} (n = 8) littermate mice for tone burst stimuli at 8, 12, 20 and 26 kHz. ABR thresholds of *Scn8a*^{Clth} / + were significantly different from +/+ at 8 kHz (†, P = <0.05). *Scn8a*^{Clth} / *Scn8a*^{Clth} ABR thresholds were significantly different from +/+ at all test frequencies (*, P = <0.05). (**b**) Representative ABR traces from 5 to 7-month old +/+, *Scn8a*^{Clth} / + and *Scn8a*^{Clth} / *Scn8a*^{Clth} mice at 80, 70, 60 and 50 dB sound pressure level (SPL) in response to 8 kHz tone burst stimuli. Peaks I–V of each ABR response trace are labeled with white squares. At 60 and 50 dB SPL, a response is seen from *Scn8a*^{Clth} / *Scn8a*^{Clth} mice, but peak morphology is altered and amplitude reduced in comparison to +/+ and *Scn8a*^{Clth} / + mice. Adapted and reprinted, with permission, from reference 15.

different components of the peripheral auditory pathway (see above). We found that ABR peak latency between peaks 3 and 5 is prolonged in *Scn8a*^{Clth}/*Scn8a*^{Clth} mice and to a lesser degree in *Scn8a*^{Clth}/+ mice. Peaks 3, 4 and 5 of the ABR are thought to correspond to the retro-cochlear neural regions of the auditory pathway[15]. Finally, to confirm that the hearing loss in *Cloth-ears* mice was not caused by dysfunction of the cochlear amplifier and that OHC function was normal in these mice, we measured DPOAEs in 6-month-old *Scn8a*^{Clth}/*Scn8a*^{Clth} and *Scn8a*^{Clth}/+ mice. DPOAE thresholds were not significantly different from wild-type mice indicating that normal OHC function is preserved in *Cloth-ears* mice. Mapping and positional candidate gene analysis of the Cloth-ears mutant identified and confirmed a point mutation in the neuronal voltage-gated sodium channel alpha-subunit gene, *Scn8a*, causing an aspartic acid to valine (D981V) change six amino acids downstream of the sixth transmembrane segment of the second domain[15]. Overall, our phenotyping pipeline identified and characterized a new deafness mutant carrying a defect in higher peripheral auditory function and demonstrated that the cochlear function is normal.

In summary, ENU mutagenesis coupled with a robust auditory and vestibular phenotyping pipeline is a powerful tool for identifying novel auditory and vestibular mutants and provides new insights into the relationship between gene and phenotype in this disease system. The application of these protocols and approaches will be important for further dissection of genes and genetic pathways involved in hearing loss.

ACKNOWLEDGMENTS This work was supported by the MRC and FP6 Integrated Project EUROHEAR, LSHG-CT-2004-512063. We thank Steve Thomas and Kevin Glover for figure preparation.

AUTHOR CONTRIBUTIONS R.E.H.-H. and S.D.M.B. designed the pipeline; R.E.H.-H. and A.P. carried out experimental work utilizing the pipeline; and R.E.H.-H., A.P. and S.D.M.B. wrote the paper.

Published online at http://www.natureprotocols.com/.
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/.

1. Brown, S.D., Hancock, J.M. & Gates, H. Understanding mammalian genetic systems: the challenge of phenotyping in the mouse. *PLoS. Genet.* **2**, e118 (2006).
2. Brown, S.D., Hardisty-Hughes, R.E. & Mburu, P. Quiet as a mouse: dissecting the molecular and genetic basis of hearing. *Nat. Rev. Genet.* **9**, 277–290 (2008).
3. Brown, S.D. & Hardisty, R.E. Mutagenesis strategies for identifying novel loci associated with disease phenotypes. *Semin. Cell Dev. Biol.* **14**, 19–24 (2003).
4. Acevedo-Arozena, A. *et al.* ENU mutagenesis, a way forward to understand gene function. *Annu. Rev. Genomics Hum. Genet.* **9**, 49–69 (2008).
5. Barbaric, I. *et al.* An ENU-induced mutation in the Ankrd11 gene results in an osteopenia-like phenotype in the mouse mutant Yoda. *Physiol. Genomics* **32**, 311–321 (2008).
6. Hough, T.A. *et al.* Novel mouse model of autosomal semidominant adult hypophosphatasia has a splice site mutation in the tissue nonspecific alkaline phosphatase gene Akp2. *J. Bone Miner. Res.* **22**, 1397–1407 (2007).
7. Buchner, D.A., Seburn, K.L., Frankel, W.N. & Meisler, M.H. Three ENU-induced neurological mutations in the pore loop of sodium channel Scn8a (Na(v)1.6) and a genetically linked retinal mutation, rd13. *Mamm. Genome* **15**, 344–351 (2004).
8. Hart, A.W. *et al.* Genotype-phenotype correlation of mouse pde6b mutations. *Invest. Ophthalmol. Vis. Sci.* **46**, 3443–3450 (2005).
9. Thaung, C. *et al.* Novel ENU-induced eye mutations in the mouse: models for human eye disease. *Hum. Mol. Genet.* **11**, 755–767 (2002).
10. Hough, T.A. *et al.* Novel phenotypes identified by plasma biochemical screening in the mouse. *Mamm. Genome* **13**, 595–602 (2002).
11. Inoue, M. *et al.* A series of maturity onset diabetes of the young, type 2 (MODY2) mouse models generated by a large-scale ENU mutagenesis program. *Hum. Mol. Genet.* **13**, 1147–1157 (2004).
12. Bacon, Y. *et al.* Screening for novel ENU-induced rhythm, entrainment and activity mutants. *Genes. Brain Behav.* **3**, 196–205 (2004).
13. Beckers, J., Wurst, W. & de Angelis, M.H. Towards better mouse models: enhanced genotypes, systemic phenotyping and envirotype modelling. *Nat. Rev. Genet.* (2009).
14. Sharpe, J. *et al.* Optical projection tomography as a tool for 3D microscopy and gene expression studies. *Science* **296**, 541–545 (2002).
15. Mackenzie, F. *et al.* Analysis of the mouse mutant Cloth-ears shows a role for the voltage-gated sodium channel Scn8a in peripheral neural hearing loss. *Genes Brain Behav.* **8**, 699–713 (2009).
16. Justice, M.J. *et al.* Effects of ENU dosage on mouse strains. *Mamm. Genome* **11**, 484–488 (2000).
17. Tsai, H. *et al.* The mouse slalom mutant demonstrates a role for Jagged1 in neuroepithelial patterning in the organ of Corti. *Hum. Mol. Genet.* **10**, 507–512 (2001).
18. Rogers, M.J. *et al.* Genetic mapping of the whirler mutation. *Mamm. Genome* **10**, 513–519 (1999).
19. Rogers, D.C. *et al.* Behavioral and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment. *Mamm. Genome* **8**, 711–713 (1997).
20. Sawada, I., Kitahara, M. & Yazawa, Y. Swimming test for evaluating vestibular function in guinea pigs. *Acta. Otolaryngol. Suppl.* **510**, 20–23 (1994).
21. Nolan, P.M. *et al.* A systematic, genome-wide, phenotype-driven mutagenesis programme for gene function studies in the mouse. *Nat. Genet.* **25**, 440–443 (2000).
22. Zheng, Q.Y., Johnson, K.R. & Erway, L.C. Assessment of hearing in 80 inbred strains of mice by ABR threshold analyses. *Hear. Res.* **130**, 94–107 (1999).
23. Jewett, D.L. & Romano, M.N. Neonatal development of auditory system potentials averaged from the scalp of rat and cat. *Brain Res.* **36**, 101–115 (1972).
24. Buchwald, J.S. & Huang, C. Far-field acoustic response: origins in the cat. *Science* **189**, 382–384 (1975).
25. Huang, C.M. & Buchwald, J.S. Interpretation of the vertex short-latency acoustic response: a study of single neurons in the brain stem. *Brain Res.* **137**, 291–303 (1977).
26. Brown, S.D., Chambon, P. & de Angelis, M.H. EMPReSS: standardized phenotype screens for functional annotation of the mouse genome. *Nat. Genet.* **37**, 1155 (2005).
27. Hardisty-Hughes, R.E. *et al.* A mutation in the F-box gene, Fbxo11, causes otitis media in the Jeff mouse. *Hum. Mol. Genet.* **15**, 3273–3279 (2006).
28. Zheng, Q.Y., Hardisty-Hughes, R. & Brown, S.D. Mouse models as a tool to unravel the genetic basis for human otitis media. *Brain Res.* **1091**, 9–15 (2006).
29. Hardisty, R.E. *et al.* The deaf mouse mutant Jeff (Jf) is a single gene model of otitis media. *J. Assoc. Res. Otolaryngol.* **4**, 130–138 (2003).
30. Parkinson, N. *et al.* Mutation at the Evi1 locus in Junbo mice causes susceptibility to otitis media. *PLoS Genet.* **2**, e149 (2006).
31. Rhodes, C.R. *et al.* The homeobox gene Emx2 underlies middle ear and inner ear defects in the deaf mouse mutant pardon. *J. Neurocytol.* **32**, 1143–1154 (2003).
32. Steel, K.P., Barkway, C. & Bock, G.R. Strial dysfunction in mice with cochleo-saccular abnormalities. *Hear Res.* **27**, 11–26 (1987).
33. Holme, R.H., Kiernan, B.W., Brown, S.D. & Steel, K.P. Elongation of hair cell stereocilia is defective in the mouse mutant whirler. *J. Comp. Neurol.* **450**, 94–102 (2002).
34. Erven, A. *et al.* A novel stereocilia defect in sensory hair cells of the deaf mouse mutant Tasmanian devil. *Eur. J. Neurosci.* **16**, 1433–1441 (2002).
35. MacArthur, C.J., Hefeneider, S.H., Kempton, J.B. & Trune, D.R. C3H/HeJ mouse model for spontaneous chronic otitis media. *Laryngoscope* **116**, 1071–1079 (2006).
36. Zheng, Q.Y., Tong, Y.C., Alagramam, K.N. & Yu, H. Tympanometry assessment of 61 inbred strains of mice. *Hear. Res.* **231**, 23–31 (2007).
37. Smith, A.P. *et al.* Analysis of breeding and pathology helps refine management practices of a large-scale *N*′-ethyl-*N*′-nitrosourea mouse mutagenesis programme. *Lab. Anim.* **43**, 1–10 (2009).