Analysis of the mouse mutant Cloth-ears shows a role for the voltage-gated sodium channel Scn8a in peripheral neural hearing loss

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Deafness is the most common sensory disorder in humans and the aetiology of genetic deafness is complex. Mouse mutants have been crucial in identifying genes involved in hearing. However, many deafness genes remain unidentified. Using N-ethyl N-nitrosourea (ENU) mutagenesis to generate new mouse models of deafness, we identified a novel semi-dominant mouse mutant, Cloth-ears (Clth). Cloth-ears mice show reduced acoustic startle response and mild hearing loss from ~30 days old. Auditory-evoked brainstem response (ABR) and distortion product otoacoustic emission (DPOAE) analyses indicate that the peripheral neural auditory pathway is impaired in Cloth-ears mice, but that cochlear function is normal. In addition, both Clth/+ and Clth/+ mice display paroxysmal tremor episodes with behavioural arrest. Clth/+ mice also suggest a novel role for Scn8a in peripheral neural hearing loss and paroxysmal motor dysfunction.

Keywords: Deafness, mouse, paroxysmal motor, peripheral neural hearing loss, Scn8a, Nav1.6, tremor, VGSC

Received 10 October 2008, revised 15 January 2009, 9 March 2009 and 1 June 2009, accepted for publication 5 June 2009

Profound or severe hearing impairment affects around 1 in 1000 live births and another 1 in 1000 children suffer hearing loss before adulthood. Approximately 30% of humans over the age of 65 and 40–50% of humans over the age of 75 are affected by hearing loss [National Institute on Deafness and Other Communication Disorders (NIDCD, http://www.nidcd.nih.gov)]. Defects in the external, middle and inner ears and the peripheral neural and central auditory processing systems can result in hearing impairment in mice and humans. Pathological causes include morphological defects of the middle and inner ears, developmental and functional defects of cochlear components and neural defects in peripheral audition and central auditory processing (Steel 1995). Genetic factors are estimated to cause around 50% of all deafness cases (Cohen & Gorlin 1995); therefore, uncovering the genetic pathways involved in hearing and deafness is crucial to the development of therapies that either prevent or correct deafness causing pathologies (Atar & Avraham 2005; Holley 2005). However, the aetiology of genetic deafness is complex. A large number of genes involved in hearing and/or deafness have been identified (see Hereditary Hearing Loss homepage: http://webh01.ua.ac.be/hhh/). Estimates vary but it seems likely that several hundred genes may be involved in the hearing process. Not only have mouse models been crucial in identifying many of these genes, but in addition many mouse mutants have recapitulated the variety of pathologies, age of onset and mode of inheritance of human deafness (Brown et al. 2008). Comparison of human deafness loci and the map positions of known deaf mouse mutants shows that many loci do not have a comparative locus in the other species (Parkinson & Brown 2002), emphasizing that a large number of genes remain to be discovered. In particular, the genetics of peripheral neural hearing, central auditory processing, late-onset and noise-induced hearing loss is still poorly understood.
To identify novel genes involved in hearing loss, we used the resources of the UK N-ethyl N-nitrosourea (ENU) mouse mutagenesis programme (Nolan et al. 2000). One mutant generated from this screen, the Cloth-ears (Clth) mouse, was identified by its poor startle response to sound. We found that Clth/Clth mice have mild but discernable hearing loss [+12 dB sound pressure level (SPL) threshold shift]. Auditory-evoked brainstem response (ABR) and distortion product otoacoustic emission (DPOAE) analysis indicated that this hearing loss is of peripheral (retrocochlear) origin. Furthermore, the Cloth-ears mutant has additional motor symptoms. Mapping and candidate gene sequencing showed a novel mutation in the neuronal voltage-gated sodium channel (VGSC) α-subunit Scn8a, causing a non-synonymous change in the SCN8A peptide. The Scn8a gene is mutated in at least 11 mouse mutants, which show a range of motor dysfunction including paralysis, dysartria and tremor. However, mutation in Scn8a has never been shown to cause hearing impairment in either mice or humans. Here, we show that mutation in Scn8a is associated with peripheral neural hearing impairment and paroxysmal motor dysfunction in a novel mouse mutant and suggest a role for SCN8A in these processes.

Methods

Animals and complementation testing

Mice were kept on a 12-h-light, 12-h-dark cycle at 19–23°C and 45–65% humidity. Up to four mice were housed per cage. Mice were fed SDS 3ME (Special Diet Services, Witham, Essex, UK) pellets and 45–65% humidity. Up to four mice were housed per cage. Mice were once a genetic map position for Cloth-ears was established. The Cloth-ears founder arose on a BALB/cAnN × C3H/HeH genetic background. Cloth-ears mice were generated by backcross breeding of genotyped Clth/+ to C3H/HeH or by intercross breeding of genotyped Clth/+ mice. In vitro fertilization (IVF) procedures were carried out by the MRC Harwell Frozen Embryo and Sperm Archive (FESA) core. For complementation testing, cryptopreserved sperms from C57BL/J6Scn8a+/+ mice carrying the N1370T mutation in Scn8a was obtained from the Jackson Laboratory (stock number 004102) and redewed by IVF and maintained by backcross on the C57BL/6J strain. Several C57BL/J6-Scn8a+/+ mice were mated to confirmed Scn8a+/+ mice, and the offspring from several litters were examined for abnormal phenotypes (including tremor, gait defects, hindlimb dragging and paralysis) every 2 days from birth until 21 days old, when mice were culled for welfare reasons.

Genetic mapping

DNA extraction from mouse tissues and PCR amplification were performed under standard protocols. Annotated polymorphic markers were identified from public databases [microsatellite markers: Mouse Genome Informatics (MGI), Broad Institute Genetic Map of the Mouse Genome (Whitehead/MIT) and Center for Inherited Disease Research (CIDR); single nucleotide polymorphism (SNP) markers: Ensembl, Genomics Institute of the Novartis Research Foundation website (GNFI) and were tested for polymorphism in the parental strains BALB/cAnN and C3H/HeH. Polymerase chain reaction of microsatellite amplicons for low-resolution mapping was performed using one fluorescently labelled primer per reaction. Polymerase chain reaction products were analysed on 6% acrylamide gels on an ABI Prism 377 DNA sequencer and 377 XL DNA sequencer Data Collection (version 2.6) software. Results were analysed using ABI Prism GeneScan analysis software (version 3.7.1) and ABI Prism Genotyper software (version 3.7). For high-resolution mapping, polymorphic microsatellite and SNP amplicons were analysed on 6% acrylamide gels using the single-stranded conformational polymorphism (SSCP) method and by pyrosequencing on a PSQ HS 96A pyrosequencer (Biotage) using one biotinylated primer per reaction. Primers used for the in-house marker Scn8a SNP were forward: AGCTAATAAGCGAGGAGG, reverse: CACCTATGACTAAGGAGC and Scn8a_SNP: CAGCACCGCCCTATGTCTCTTT.

Candidate gene assessment and mutation screening

Candidate genes mapping to the non-recombinant region were identified using the Ensembl mouse genome database (versions 30, 32 and 33) and assessed for candidacy by expression and function [MGI, Genecards database, version 2.31 (Weizmann Institute of Science)], mouse mutants [MGI, Transgenic/Targeted Mutation database (TBASE)], and associated human disorders [Online Mendelian Inheritance of Man (OMIM)], Exonic and splice-site sequence from candidate genes was amplified from genomic DNA from two Clth/Clth mice and the two parental strains BALB/cAnN and C3H/HeH, and was sequenced under standard protocols. Sequences were examined for integrity using the ABIPrism EditView ABI Automated DNA Sequence Viewer (version 1.0.1) viewer programme and analysed for mutation using DNASTar Lasergene (v6.0) sequence analysis software.

Peptide predictions

Peptide sequences of mouse SCN8A, other mouse SCN-alpha peptides and SCN8A orthologues were obtained from Ensembl. Wild-type and mutant Cloth-ears peptide sequences were predicted and compared using EditSeq computer software (DNASTar). Other mouse SCN peptides and SCN8A orthologues were aligned using clustal-W web-based software (http://www.ebi.ac.uk/Tools/clustalw/). The position of the Cloth-ears mutant amino acid in the SCN8A protein was determined using putative predicted protein domains from Ensembl and Uniprot databases.

Analysis of auditory function

Assessment of the acoustic startle response

Clickbox (Institute of Hearing Research, Nottingham, UK) and quantitative startlebox (acoustic startle) (SmithKline Beecham Pharmaceuticals, plc) protocols were carried out as outlined on the EMPRESS website (www.empress.har.mrc.ac.uk). During clickbox testing, a quick backwards flick of the ear pinna (the Preyer’s reflex) and a rapid ‘jump’ or contraction of the neck and trunk muscles (the startle response) was recorded as a normal response. A lack of either the Preyer’s reflex or startle response, or a markedly milder response of either reflexes, was recorded as a reduced response. A complete lack of both reflexes was recorded as no response. In startlebox (acoustic startle) analysis, the maximal startle amplitude and the latency of the maximal startle to 110 dB SPL, white noise, 40 ms tone bursts, and of background movement, was recorded. Mean gross startle amplitudes were calculated from 10 startle trials, mean basal movement was calculated from 10 recordings in the absence of a soundburst and mean startle amplitudes were calculated by subtracting the mean basal movement from the mean gross startle amplitude for each mouse. Amplitude and latency of the startle response of mice was measured using Windows 3.1 software.

Auditory-evoked brainstem response (ABR) analysis

Mice were anaesthetized with ketamine (Ketaset®) and additionally given medetomidine (Domitor®) for muscle relaxation and analgesia by intraperitoneal injection (0.5 ml Domitor® at 100 mg/ml with 4.12 ml water and 0.38 ml Ketaset® at 1 mg/ml; administered at a rate of 0.1 ml/10 g of body weight). Animals were placed in an
audiometric chamber (IAC 401-A-SE) on a heated mat (~37°C) to maintain body temperature. Acoustic stimuli were delivered monaurally to the right ear at a distance of 1.5 cm via a free field transducer (ESI Tucker Davis Technology (TDT), Alachua, FL, USA), controlled by SigGen/BioSig software (TDT), using TDT system III hardware. The transducer was calibrated using a 1/2" measuring microphone (7016 ACO-Pacific, Belmont, CA, USA) and SigCal software (TDT). Electrodes (Grass Telefactor F-E2-12) were placed subcutaneously over the vertex (active), right mastoid (reference) and left mastoid (ground). The response from the electrodes was recorded for a period of 10 ms, over a total of 312 repetitions, and amplified using TDT system III hardware. The repetitions were averaged and bandpass filtered between 300 and 1500 Hz, using the BioSig software. Tone burst stimuli totaled 7 ms duration including averaged and bandpass filtered between 300 and 1500 Hz, using the TDT system III hardware. The repetitions were recorded for a period of 10 ms, over a total of 312 repetitions, and left mastoid (ground). The response from the electrodes was placed subdermally over the vertex (active), right mastoid (reference) and gold (ground). The field transducer (ES1 Tucker Davis Technology (TDT), Alachua, FL, USA) was used for sound system calibration and acoustic stimulation. Data were digitized at 250 kHz and stored on a PC. Distortion product otoacoustic emission threshold curves were constructed from measurements of the level of the f1 tone that produced a 2f1-f2 DPOAE with a level of 0 dB SPL, where the frequency ratio of f2/f1 = 1.23. System distortion during DPOAE measurements was 80 dB below the primary tone levels.

Histological analysis
All mice examined by histological methods were sex matched with littermate controls. Ossicles were dissected, placed into formalin solution and examined by X-ray using Faxitron equipment (Qados Ltd, Sandhurst, Berkshire, UK). For examination of cochlear hair cells, brains were removed and bisected heads were placed into 2.5% glutaraldehyde in 0.1 M phosphate buffer. Cochlear hair cells were dissected and fixed in fresh 2.5% glutaraldehyde in 0.1 M phosphate buffer for 4–5 h, rotating, at 4°C, and then washed four times for 5 min in 0.1 M phosphate buffer in buffer. The bony shell of the cochlea was then removed. Samples were processed by dehydration, critical point drying and gold sputter coating at the Electron Microscopy Unit, Royal Holloway University of London, Egham, Surrey TW20 0EX. Ultrastructural analysis of cochlear hair cells was carried out using Hitachi S-2400 and S-3000N scanning electron microscopes. Nocropcy and histological analysis was carried out as outlined on the EMPReSS website (www.empress.har.mrc.ac.uk). For cerebellar histopathology, brains were removed and bisected heads were placed into 4% PFA (in PBS, pH 7.4) and examined by X-ray analysis using Faxitron equipment. For examination of Purkinje cell layer, brains were removed and bisected heads were placed in 4% PFA (in PBS, pH 7.4) and stained with cresyl violet. Five micrometre parasagittal cryosections were cut on a microtome, mounted on Superfrost Plus slides (BDH) and stained in 0.5% cresyl violet solution. For cell counts and measurements, 5 μm coronal wax sections from fixed brains were taken every 100 μm through the cerebellum and stained with cresyl violet. Five micrometre sections (spanning the sections was used for cell counts and measurements at three regions in the anterior (containing predominantly lobes I–VI), posterior (lobes VII–IX) and midline between these selected regions, posterior to the primary fissure (lobes VI and X). Cells were counted if the nuclei could be visualized. Maximum width of the sections (discounting the parafolliculus) and distance from the top of the cerebellum to the fourth ventricle or medulla at the base of the cerebellar vermis were also measured. The ratio between the width Purkinje cell layer (PCL) and granule cell layer (GCL) was calculated by measuring matched regions in the cerebellar hemispheres in all sections.

Phenotypic and behavioural testing
A cohort of 73 +/+ Scn8a<sup>Chm</sup> +/– and Scn8a<sup>Chm</sup> Scn8a<sup>Chm</sup> mice was generated by mating nine Scn8a<sup>Chm</sup> +/– siblings (generation N5xN5N2) in six trio matings and comprised of all mice born over several phenotypes. Phenotypic analysis was carried out using a modified SHIRPA protocol, adapted from Rogers et al. (1997). A similar protocol can be found at www.empress.har.mrc.ac.uk. Additional phenotyping analysis included (1) specific observations of mouse general behaviour over 1 min (assessing the presence of facial twitches, muscle spasms of the body, piloerection, shaky or unsteady movement, abnormal postures, repetitive nose poking, hyperactivity, excess rearing); (2) fore- and back-leg grip strength using a grip strength meter (BioSeb) and (3) excess freezing behaviour occurring outside the viewing jar and the duration of freezing after the click box test. Due to welfare issues concerning Scn8a<sup>Chm</sup> +/– and Scn8a<sup>Chm</sup>/– mice, only +/+ and Scn8a<sup>Chm</sup> +/– mice were tested in the grip strength test (n = 56). Scoring of tests shown in Fig. 5 was performed as follows: wire manoeuvre: good attempt = 1, impaired attempt = 0; limbgrasping: absent = 0, present = 1; toe-pincher reflex: absent = 0, present = 1 and freezing during testing: absent = 0, present = 1. Thirty nine +/+, Scn8a<sup>Chm</sup> +/– and Scn8a<sup>Chm</sup>/– littermate mice from the cohort were tested in the open-field and light-dark behavioural tests (five females and eight males per genotype). Open-field testing was performed as outlined on the EMPReSS website (www.empress.har.mrc.ac.uk) using videotracking software analysis by EthoVision; mice were scored for latency of first movement in arena (seconds), number of movements into the centre, duration spent in centre (seconds), distance moved in arena (cm) and in centre (cm), maximum distance per move in arena (cm) and in centre (cm), mean velocity in arena (cm/seconds) and in centre (cm/seconds), duration of moving in arena (seconds) and in centre (seconds). Light-dark apparatus and software used was TruScan 99 (Coulbourne Instruments). Tests were performed between 0900 and 1400 h. Mice were tested in the same order as the open-field test. Mice were moved from the home room into a quiet acclimatization room at least 30 min before the start of the test. A clean, opaque Perspex dark box was placed in the left-hand side of the arena with the opening facing to the left. Red light only was used for illumination using a lamp directly above the ‘lit’ region of the arena. Mice were placed directly from the home cage into the opening of the dark box, and the recording was started. Mice time for the mouse to enter the light half of the arena (scored by all four paws placed in the light half) was recorded manually and the mouse was left undisturbed to complete the test.

Statistical analysis
Except for ABR threshold data, which were analysed using a two-tailed Student’s t-test, statistical analysis was carried out using the software package R (2008). Data was statistically analysed for a genotype effect. To adjust for confounding variables, continuous numeric data were analysed under a linear model using analysis of variance (ANOVA) and multivariate F-test; binary data were analysed under a generalized linear model and ordinal data were analysed under a proportional odds logistic regression model. The significance of a genotype effect was tested under a model which accounted...
for other factors, namely, (mating identifier, sex, genotype) vs. (mating identifier, sex). Data obtained by using different mouse cohorts were analysed separately. Tests showing an ‘imbalance of design’ where there was a large discrepancy in the number of individuals showing different responses within a test, e.g. in a binary (qualitative) test where 65 mice showed a positive response and 8 mice showed a negative response] were manually analysed for the distribution of values by genotype. P values were corrected for multiple hypotheses testing using the false discovery rate test (FDR, 5%). Estimate values for Scn8a<sup>Clth/+</sup> and Scn8a<sup>Clth/Clth</sup> mice (relative to wild-type) were further tested to find the best fit of mode of inheritance, under a ‘dominant’ model [(mating, sex, homozygous status, wild-type status)] and a ‘recessive’ model [(mating, sex, non-homozygous status, wild-type status)] against (mating, sex, non-wild-type status) and (mating, sex, non-homozygous status) vs. (mating, sex, non-wild-type status, homozygous status).

**Results**

**Identification of the Cloth-ears mouse mutant**

The Cloth-ears mouse mutant was identified in a dominant mouse ENU-mutagenesis screen (Nolan et al. 2000). The founder mutant presented at 5 weeks with a reduced startle response to a 90 dB SPL, 20 kHz single tone burst, generated by a clickbox. Frozen sperm from the founder mouse was used to fertilize C3H/HeH oocytes by IVF. Startle response in offspring was assessed by clickbox test. We found that 30% of N3 offspring (n = 66) showed a mild or absent Peyer’s reflex and a mild or absent startle response, confirming the genetic basis of this phenotype. A colony of Cloth-ears mice was generated for genetic mapping and phenotypic analysis by backcross of confirmed Cloth/+ mice to the C3H strain. Phenotype analysis of an N4 generation backcross cohort showed that the reduced startle response was autosomal and inherited, but at reduced penetrance (16 of 58 backcross mice). In addition, Cloth/+ mice showed an episodic tremor (see below). Cloth-ears/+ mice with a reduced startle response were intercrossed to produce Cloth/Cloth mice. Putative Cloth/Cloth mice displayed a continuous tremor (see below) and also displayed a reduced startle response to the clickbox, but at much greater penetrance (5 of 23 intercross mice). Cloth/+ and Cloth/Cloth mice showed a normal lifespan, no unexpected lethality and were fertile. The severity of continuous tremor in Cloth/Cloth mice and the frequency and severity of episodic tremor in Cloth/+ mice appeared to increase moderately with age (< 2 years), but neither Cloth/+ or Cloth/Cloth mice developed gait defects, limb weakness or paralysis at any age.

**Genetic mapping and identification of the Cloth-ears gene**

The Cloth-ears mutation arose on a mutagenized (BALB/cAnN × C3H/HeH)F1 individual. Marker analysis of 64 affected Cloth/+ backcross progeny allowed us to localize the Cloth-ears mutation to a 3.1 Mb region on distal chromosome 15 between the microsatellite markers D15Mit42 and D15Mit246 (Ensembl, version 50) (Fig. 1a), and indicated that the mutagenized chromosome was from the BALB/c strain. Moreover, genetic analysis of Cloth/Cloth mice derived from Cloth/+ intercrosses and showing a continuous tremor refined the non-recombinant region to 1.37 Mb between the markers D15Mit97.2 and D15Mit246. Additional genotyping of non-affected (non-tremoring) mice from Cloth/+ × Cloth/+ intercrosses yielded two more recombinants that further refined the region to 1.2 Mb between the markers D15Mit97.2 at 100.63 Mb and rs8266857 at 101.83 Mb (Fig. 1a). The Cloth-ears locus was non-recombinant with the marker rs4231023 and the in-house marker Scn8a<sub>SNP</sub>. The Cloth-ears non-recombinant region contains 33 genes, including a cluster of keratin genes and several novel genes.

**Cloth-ears does not complement a known mutant of Scn8a**

To assess whether the D981V mutation in SCN8A was causing the Cloth-ears phenotype, we carried out a
Mutation in Scn8a in deafness and paroxysmal tremor

Figure 1: (a) Haplotypes of backcross and intercross progeny segregating the Cloth-ears mutation. The Cloth-ears (Clth) mutation arose on a mutagenized (BALB/cAnN × C3H/HeH)F1 individual. Sixty-four backcross progeny with a reduced startle response and/or an intermittent tremor and 26 intercross progeny with or without a continuous tremor were mapped across chromosome 8 and genotyped at recombinant markers. The genetic data indicates that the mutagenized chromosome was from the BALB/cAnN strain. The Cloth-ears mutation is non-recombinant with the SNP markers Scn8a_SNP and rs4231023 and maps to the interval D15Mit97.2–rs8266857.

(b) Chromatograms of the Cloth-ears mutation from wild-type (+/+) and Scn8aClth/Scn8aClth DNA. Arrow: base 2942 of the Scn8a coding sequence, showing an adenine (A) in wild-type mice and a thymine (T) in Scn8aClth/Scn8aClth mice (arrow). The predicted amino acid sequence from wild-type and Scn8aClth/Scn8aClth mice is also indicated, highlighting the D981V change. (c) Position of the Cloth-ears mutation (D981V) in the SCN8A amino acid sequence. D981V resides within the predicted domain 2 (D2) of the SCN8A peptide, six amino acids downstream of the S6 transmembrane segment. The amino acid sequence of domain 2 (D2) is shown in black; sequence of domain 2 segment 6 (D2S6) is shown in pink and the position of the Cloth-ears mutation (D981V) is shown in red. (d) Schematic of the voltage-gated sodium channel α- and β-subunit proteins (adapted from Meisler & Kearney 2005). The α-subunit comprises four homologous domains (D1–D4 or D1–DIV), which are shown in different colours, each containing six transmembrane segments (S1–S6). S4 segments function as voltage sensors and the S5–S6 loops form the outer pore. A pore structure is formed by the transmembrane segments in the membrane (inset). The location of the Cloth-ears mutation is shown (red star). (e) Alignment of peptide sequences of SCN8A orthologues and other mouse SCN α-subunits (Ensembl) in the region of amino acid 981 (arrow). Alignments were made using clustal-W web-based software.

A complementation test cross with Scn8a4J, a known mouse mutant of Scn8a. The Scn8a4J mutation is a missense mutation (Buchner et al. 2004). However, Scn8a4J/Scn8a4J mice display a much more severe phenotype, showing hindlimb paralysis and juvenile death. Scn8a4J/+ mice are not reported to show any abnormal phenotype (Buchner et al. 2004). Clth/+ mice were crossed to Scn8a4J/+ mice. Of 15 offspring, 3 showed a continuous tremor in the head and body (20%), beginning at 8–9 days old. Extended freezing and severe tremor episodes were noticeably present in one mouse. Interestingly, these mice also showed an abnormal gait. This result shows that Cloth-ears and Scn8a4J do not complement: Clth+/+ Scn8a4J/+ mice show a phenotype that clearly resembles the Clth/Clth phenotype, but that is
slightly more severe. The Cloth-ears mutant will be therefore referred to as an allele of Scn8a, Scn8aCloth.

**Cloth-ears mice have a reduced acoustic startle response**

We assessed in more detail the onset of startle response impairment in Scn8aCloth/+ mice by weekly clickbox tests of mice from an age-matched backcross cohort (n = 58). The onset of a persistently reduced startle response was found to be between 31 and 79 days (Fig. 2a). We assessed the impairment of the startle response quantitatively using startlebox analysis. At 6, 10 and 18 weeks old, both Scn8aCloth/+ and Scn8aCloth/Scn8aCloth mice showed significantly reduced amplitude of startle response to white noise at 110 dB SPL. Statistical analysis showed that the reduction in startle best fit a dominant model (see Methods; Fig. 3a,c). This result confirmed the reduced startle response that was observed in Scn8aCloth mice by clickbox test. In addition, the startle amplitude progressively worsened between 6 and 10 weeks old (Fig. 3c). This suggests that the impairment of startle in Scn8aCloth mice begins before 6 weeks of age and that this impairment progresses until at least 10 weeks of age, supporting the previous findings from weekly clickbox tests. No difference in latency of peak startle amplitude was found at 6, 10 or 18 weeks of age in either Scn8aCloth/+ or Scn8aCloth/Scn8aCloth mice (Fig. 3a). A residual startle response in almost all mice tested indicated that neither Scn8aCloth/+ or Scn8aCloth/Scn8aCloth mice were profoundly deaf. However, this analysis did not rule out the possibility of a motor impairment, instead of hearing loss, as the primary cause of the reduced startle response. In
addition, Scn8a<sup>Cth</sup>/+ or Scn8a<sup>Cth</sup>/Scn8a<sup>Cth</sup> mice did not show evidence of vestibular impairments such as circling, headbobbing or abnormal behaviour in grid walking or swimming (data not shown).

**Cloth-ears mice display ABR abnormalities indicative of a peripheral neural auditory defect**

We examined Cloth-ears mice for hearing loss by ABR analysis. To determine auditory thresholds, ABR analysis was performed on sex-matched 3-month-old +/-, Scn8a<sup>Cth</sup>/+ and Scn8a<sup>Cth</sup>/Scn8a<sup>Cth</sup> mice (n = 22) at decreasing dB SPL levels from 90 dB SPL at 8, 12, 20 and 26 kHz (Fig. 2c). Statistical analysis showed that auditory thresholds of Scn8a<sup>Cth</sup>/+ mice were only significantly increased compared with +/- mice at 8 kHz, but that Scn8a<sup>Cth</sup>/Scn8a<sup>Cth</sup> thresholds were significantly increased compared with +/- mice across the four frequencies tested (see figure legend). Scn8a<sup>Cth</sup>/Scn8a<sup>Cth</sup> mice showed an average threshold increase of 10–14 dB SPL. This indicates that Scn8a<sup>Cth</sup> mice have mild semi-dominant hearing loss.

To investigate the pathological causes of hearing loss in Scn8a<sup>Cth</sup> mice, we examined auditory structures including the outer, middle and inner ears and the peripheral neural auditory pathway. Dissection and X-ray analysis of the middle ear ossicles of 6–10-month-old Scn8a<sup>Cth</sup>/+ (n = 2) and Scn8a<sup>Cth</sup>/Scn8a<sup>Cth</sup> (n = 2) mice showed no malformations compared to controls (Fig. 2b). Haemotoxylin and eosin (H&E)-stained sections of middle ears from 8-month-old Scn8a<sup>Cth</sup>/+ (n = 2) and Scn8a<sup>Cth</sup>/Scn8a<sup>Cth</sup> (n = 2) mice did not show any differences to controls, with normal middle ear epithelia, intact tympanic membranes and no evidence of otitis media (Fig. 2b). H&E-stained cochlear sections from 6-month-old Scn8a<sup>Cth</sup>/+ (n = 2) and Scn8a<sup>Cth</sup>/Scn8a<sup>Cth</sup> (n = 2) mice showed no mispatterning of the stereocilia or degeneration of hair cells, two common pathologies of hearing loss in both mice and humans (Fig. 2b). Gross cochlear structure and outer ear structure in Scn8a<sup>Cth</sup>/+ and Scn8a<sup>Cth</sup>/Scn8a<sup>Cth</sup> mice were also normal (data not shown).

The lack of an obvious peripheral structural defect (in outer, middle or inner ears) in Scn8a<sup>Cth</sup> mice led us to extend our ABR analyses and focus on possible deficits in higher peripheral neural auditory regions. A typical ABR waveform is composed of five peaks, corresponding to electrical signals generated by different components of the peripheral auditory pathway. ABR analysis was performed on sex-matched 5–7-month-old +/-, Scn8a<sup>Cth</sup>/+ and Scn8a<sup>Cth</sup>/Scn8a<sup>Cth</sup> mice (n = 15) using 90 dB SPL tones at 8, 12, 20 and 32 kHz, and peak latencies were statistically analysed for differences (Figs. 2d and 3b,d). Both Scn8a<sup>Cth</sup>/+ and Scn8a<sup>Cth</sup>/Scn8a<sup>Cth</sup> mice showed no differences in latency of peak 1 (Fig. 3b,d), thought to represent...
Mackenzie et al.

A

| Parameter       | 6 weeks                              | 10 weeks                              | 18 weeks                              |
|-----------------|--------------------------------------|---------------------------------------|---------------------------------------|
| Startle amplitude | Dominantly reduced *** (F_{2,67} = 8.624) | Dominantly reduced *** (F_{2,67} = 36.482) | Dominantly reduced *** (F_{2,67} = 14.331) |
| Startle latency  | n.s.                                 | n.s.                                  | n.s.                                  |

B

| ABR peak latency | Frequency |
|-----------------|-----------|
|                 | 8kHz      | 12kHz     | 20kHz     | 32kHz     |
| P1              | n.s.      | n.s.      | n.s.      | n.s.      |
| P1-P5           | Recessively increased * (F_{2,67} = 7.337) | Recessively increased ** (F_{2,67} = 11.892) | Dominantly increased *** (F_{2,67} = 21.607) | Recessively increased ** (F_{2,67} = 12.949) |
| P3-P5           | n.s.      | Recessively increased *** (F_{2,67} = 15.201) | Dominantly increased ** (F_{2,67} = 7.977) | n.s.      |
| P3-P4           | n.s.      | n.s.      | n.s.      | n.s.      |
| P4-P5           | Recessively increased ** (F_{2,67} = 8.778) | n.s.      | Recessively increased * (F_{2,67} = 6.554) | n.s.      |

C

Startle amplitude

D

ABR peak latency

Figure 3: (a) Statistical analysis for genotypic differences of hearing phenotypes of Scn8aClth mice at 6, 10 and 18 weeks (n = 73), where F_{t,u} denotes the F-statistic on t degrees of freedom. Analysis was performed under a linear model (see Methods). Startle amplitudes and maximal latencies were measured in response to 40 ms soundbursts of white noise at 110 dB SPL using the startlebox paradigm. Parameters significantly different to wild types are given with the best fit of inheritance. n.s., not significant; *P < 0.05; **P < 0.01; ***P < 0.001 (unadjusted P values). (b) Statistical analysis for genotypic differences of ABR peak latencies of Scn8aClth mice at 5–7 months old, where F_{t,u} denotes the F-statistic on t degrees of freedom. Analysis was performed under a linear model (see Methods). Parameters significantly different to wild types are given with the best fit of inheritance. n.s., not significant; *P < 0.05; **P < 0.01; ***P < 0.001 (unadjusted P values). (c and d) Graphical representation of significantly different startle response and ABR peak latency parameters in Scn8aClth mice compared with wild types, showing data for adjusted P values < 0.05. Startle response was measured by placing mice in a soundproof chamber containing a loudspeaker to generate sound. Startle amplitude was calculated in arbitrary units using an accelerometer connected to the chamber floor to measure floor displacement upon sound presentation. Data shown are estimate values for Scn8aClth/+ and Scn8aClth/Scn8aClth mice, having adjusted for sex and mating identifier, where +/+ is normalized to 0.

Cochlea and cochlear nerve function (Henry 1979). However, Scn8aClth/Scn8aClth mice displayed abnormal waveform morphology from peak 3 compared with control mice: peaks 3 and 4 showed a merged morphology, with peak 4 showing a smaller amplitude than peak 3, and peaks 3–4 and 4–5 interpeak latencies appeared prolonged (Figs. 2d and 3b,d). In addition, peak 5 was often almost absent in Scn8aClth/Scn8aClth mice. Statistical analysis of peak latencies showed that many interpeak measurements were significantly different in Scn8aClth/+ and Scn8aClth/Scn8aClth mice compared with wild-type mice (Fig. 3b,d). We found that peak 1–5 latency was indeed significantly prolonged, suggesting a recessive model, in response to all frequencies tested (8, 12, 20, 32 kHz). Given the abnormal wave morphology of peaks 3–5, we looked in more detail at peak 3–5 latencies. Peak 3–5 and 4–5 latencies were also significantly prolonged, at

Genes, Brain and Behavior (2009) 8: 699–713
different frequencies (Fig. 3b,d), which may reflect different sensitivities of the ABR technique. Of particular interest is the significant prolongation of peak 3–5 latency at 20 kHz, which best fits a dominant inheritance model. As our statistical model did not account for semi-dominant inheritance, examination of the estimate values for this parameter suggests that a semi-dominant model may better fit these results (peak 3–5 latency: Scn8aClth/+ = 1.525 msec, Scn8aClth/Scn8aClth = 1.842 msec; Fig. 3d). Overall, these results suggest that ABR peak latency between peaks 3 and 5 is prolonged, certainly in Scn8aClth/Scn8aClth mice and probably in Scn8aClth/+ mice. Peaks 3, 4 and 5 of the ABR are thought to correspond to retrocochlear neural regions of the auditory pathway [probably the superior olivary complex, lateral lemniscus and inferior colliculus (Henry 1979)]. These analyses suggested that peripheral neural auditory function is abnormal in Scn8aClth mice. To confirm that the hearing loss in Cloth-ears mice was not caused by dysfunction of the cochlear amplifier and, hence (Robles & Ruggiero 2001) the outer hair cell function was normal in these mice, we measured DPOAEs in 6-month-old Scn8aClth/Scn8aClth (n = 4), Scn8aClth/+ (n = 6) and +/+ (n = 3) mice (Fig. 2e). Distortion product otoacoustic emission thresholds were not significantly different between the three genotypes (Fig. 2e), indicating that normal power amplification (Lukashkin et al. 2007) of the cochlear mechanical responses is preserved in Cloth-ears mice.

Cloth-ears mice display tremor and paroxysmal movement abnormalities

An additional motor phenotype was identified in Scn8aClth mice. Initially, we observed that Scn8aClth/+ mice displayed an episodic, complex phenotype of extended freezing behaviour, piloerection, a hunched posture and an intermittent side-to-side coarse tremor of the whole body. This phenotype was observed at the earliest in 25-day-old mice. Similarly to the reduced startle response, this phenotype was observed at the earliest in 25-day-old mice. Initially, we observed that Scn8aClth mice was not caused by dysfunction of the cochlear amplifier and, hence (Robles & Ruggiero 2001) the outer hair cell function was normal in these mice, we measured DPOAEs in 6-month-old Scn8aClth/Scn8aClth (n = 4), Scn8aClth/+ (n = 6) and +/+ (n = 3) mice (Fig. 2e). Distortion product otoacoustic emission thresholds were not significantly different between the three genotypes (Fig. 2e), indicating that normal power amplification (Lukashkin et al. 2007) of the cochlear mechanical responses is preserved in Cloth-ears mice.

The unusual motor symptoms and freezing behaviour in Scn8aClth mice led us to investigate the gross phenotype of Scn8aClth mice, firstly a recessive continuous tremor affecting general posture in Scn8aClth/Scn8aClth mice and secondarily a dominant episodic tremor with behavioural arrest affecting both Scn8aClth/+ and Scn8aClth/Scn8aClth mice.

We assessed if the tremor and motor abnormalities in Scn8aClth mice could be associated with muscular, cerebellar or other tissue changes. Sections of semimembranosus and quadriceps muscles from 8.5-month-old Scn8aClth/+ and Scn8aClth/Scn8aClth mice (n = 4) showed no differences to wild-type muscles in H&E-stained sections. Specifically, there was no evidence of degenerative or regenerative changes (fibre size asymmetry, pyknotic nuclei, centrally nucleated fibres; data not shown). We also examined H&E-stained cerebellar sections from 8.5-month-old Scn8aClth/+ and Scn8aClth/Scn8aClth mice (n = 4) that showed no indication of Purkinje cell loss or disorganization of the trilaminar structure (Fig. 4a). To quantify any potential abnormalities of the mutant cerebellum further, we examined sections of 8.5-month-old Scn8aClth/+ and Scn8aClth/Scn8aClth mice using cresyl violet staining (n = 4) (Fig. 4a). Cerebellar dimension and layer measurements and Purkinje cell counts showed that although cerebellum from Scn8aClth/Scn8aClth mice are slightly smaller than +/+ and Scn8aClth/+ animals and show a slightly reduced number of Purkinje cells, the ratio of the PCL to GCL width is normal, suggesting the overall structural development and maintenance of the cerebellum are not affected in Scn8aClth/Scn8aClth mice (Fig. 4b). Furthermore, no neuronal mislocalization was observed and the lamination of the cortex and hippocampus appeared normal in all sections examined, thus the small Scn8aClth/Scn8aClth cerebellar differences are likely to reflect the overall small size of the mice (Fig. 4a). H&E-stained sections of 33 other tissues and organs from 8.5-month-old Scn8aClth/+ and Scn8aClth/Scn8aClth mice (n = 4) showed no histological differences to wild-type mice (data not shown). Peripheral (somatic) nerves showed no abnormal myelinization and spinal cord sections showed no abnormalities (data not shown).

Lastly, we noticed that the response to anaesthesia in Scn8aClth mice was abnormal. During recovery from anaesthesia (ketamine/medetomidine) after ABR testing, Scn8aClth/Scn8aClth mice displayed abnormal dystonic postures (n = 5) that were not observed in Scn8aClth/+ or +/+ mice (Fig. 4c), and a longer time to regain normal movement (data not shown).

Behavioural abnormalities in Cloth-ears mice

The unusual motor symptoms and freezing behaviour in Scn8aClth mice led us to investigate the gross phenotype of Scn8aClth mice by detailed phenotypic and behavioural testing of +/+, Scn8aClth/+ and Scn8aClth/Scn8aClth littermate mice at 6, 10 and 18 weeks of age (n = 73). These time-points spanned the ages at which these phenotypes appeared to
Figure 4: (a) H&E-stained coronal sections of cerebellum from 8-month-old mice show no Purkinje cell loss in any region (scale bar, 50 μm). Histopathology of the cerebellum of 8.5-month-old mice using cresyl violet on coronal sections shows no neuronal mislocalization (scale bar, 1 mm). (b) Area and layer measurements and Purkinje cell counts of Scn8a<sup>Clth</sup> mice. Although Scn8a<sup>Clth</sup>/Scn8a<sup>Clth</sup> cerebellums are slightly smaller and show slightly reduced Purkinje cell counts compared with +/+ and Scn8a<sup>Clth</sup>/+, the Purkinje cell layer (PCL) to granule cell layer (GCL) ratio is normal, indicating normal overall cerebellar structure. Area measurements are in millimetres. (c) Abnormal dystonic postures displayed by Scn8a<sup>Clth</sup>/Scn8a<sup>Clth</sup> mice during recovery from anaesthesia were not seen in +/+ or Scn8a<sup>Clth</sup>/+ mice. Postures were maintained for up to 1 min.

Develop in Scn8a<sup>Clth</sup> mice. Data was statistically analysed for a genotype effect. To adjust for confounding variables, continuous numeric data were analysed under a linear model using ANOVA and multivariate F-test; binary data were analysed under a generalized linear model and ordinal data were analysed under a proportional odds logistic regression model (see Methods for further information). Parameters showing a significant difference were further analysed to determine the best fit of inheritance (dominant or recessive) (see Methods). This analysis showed that Scn8a<sup>Clth</sup> mice show significant dominant, recessive, and probably semi-dominant abnormalities in growth, neurological and motor function and behaviour. The weight of Scn8a<sup>Clth</sup> mice was significantly less than wild type at all ages tested (Fig. 5a,c), with statistical analysis suggesting both recessive (6 weeks, 10 weeks) and dominant (18 weeks) inheritance. Estimate values for Scn8a<sup>Clth</sup>/+ mice were intermediate between wild-type and Scn8a<sup>Clth</sup>/Scn8a<sup>Clth</sup> values (Fig. 5c), suggesting that growth retardation is likely to be semi-dominant. Grip strength in (1) forelegs and (2) all legs was not different in Scn8a<sup>Clth</sup>+/+ mice at any age tested (Fig. 5a). Scn8a<sup>Clth</sup>/Scn8a<sup>Clth</sup> mice were not tested using the grip strength test for welfare reasons. Ability to perform the wire maneuvre test was recessively impaired in Scn8a<sup>Clth</sup> mice at 6, 10 and 18 weeks of age (Fig. 5a,c). Scn8a<sup>Clth</sup> mice also showed recessive increased tendency to limb grasp (Fig. 5a,c). Trunk curling during tail suspension was not successfully analysed statistically because of experimental imbalance (Fig. 5a), but individual test results showed that most Scn8a<sup>Clth</sup>/Scn8a<sup>Clth</sup> mice displayed abnormal trunk curling behaviour, instead of the splayed limbs of wild-type mice (data not shown). The toe-pinch reflex was recessively impaired in Scn8a<sup>Clth</sup> mice at all ages tested (Fig. 5a,c). However, proximal reflex tests of corneal, pinna and reaching reflexes were all normal (Fig. 5a).
We assessed general behaviour by observing mice undisturbed for 1 min (see Methods). Scn8aClth mice showed a recessive increased tendency to freeze during testing at all ages tested (Fig. 5a,c) and a recessive increased duration of freezing after the clickbox test (where wild-type mice normally freeze for 1–2 seconds) at 10 and 18 weeks (Fig. 5a,c). No significant differences between Scn8aClth mice and wild-type mice were found in facial twitching, muscle spasms of body, piloerection, shaky or unsteady movement, abnormal postures, repetitive nosepoking, visible hyperactivity or excessive rearing (data not shown).

The freezing behaviour in both Scn8aClth/+ and Scn8aClth/Scn8aClth mice raised the possibility that Scn8aClth mice could suffer high anxiety. We tested anxiety and locomotion in 5–6-month-old sex-matched Scn8aClth/+ and Scn8aClth/Scn8aClth mice (n = 39) using the open-field and light-dark box paradigms. Scn8aClth mice did not show any significant differences in any parameters tested in the open-field test, indicating no observed anxiety or locomotion defects.

**Figure 5: Legend on next page.**
Mackenzie et al.

compared with wild-type mice (Fig. 5b). However, the latency of first entrance into the lit half of the light-dark box was significantly recessively reduced in Scn8a<sup>Clth</sup>/Scn8a<sup>Clth</sup> mice (Fig. 5b,d).

**Discussion**

We report that a novel ENU-induced mouse mutant, Cloth-ears (Scn8a<sup>Clth</sup>), shows hearing loss with peripheral neural auditory impairment as well as paroxysmal motor symptoms and tremor, and that a missense mutation in Scn8a underlies this phenotype. The data suggests that SCN8A is critical for normal peripheral auditory function and may play a role in the pathogenesis of paroxysmal motor disorders.

**Scn8a<sup>Clth</sup> mice are a model of peripheral neural hearing loss**

We did not detect any morphological or functional pathology in middle or inner ears from Scn8a<sup>Clth</sup> mice by histology, ABR and DPOAE analysis, showing that the Scn8a<sup>Clth</sup> mutation does not affect middle ear or cochlear function. However, ABR analysis showed that Scn8a<sup>Clth</sup> mice have abnormal morphology of peaks 3–5 and lengthened peak latencies. Peaks 1–5 of the ABR in mice are generally considered to arise approximately from the cochlear origins (peak 1), from the cochlear nucleus (peak 2), the superior olivary complex (peak 3), the lateral lemniscus (peak 4) and the inferior colliculus (peak 5) (Henry 1979). Our findings suggested that the auditory deficit in Scn8a<sup>Clth</sup> mice is caused by a peripheral neural defect originating from the superior olivary complex, lateral lemniscus or inferior colliculus (peaks 3, 4 and 5, respectively), although these generators of the ABR are still contested (Henry 1979). This suggests that SCN8A is involved in auditory nerve and peripheral neural auditory function. SCN8A is localized to the organ of Corti and cochlear ganglionic axons in the mouse, and has been shown to be involved in the generation and regeneration of action potentials in cochlear ganglion cells (Hossain et al. 2006). Interestingly, a role for Scn8a in the central auditory pathway was previously suggested (Chen et al. 1999) by observation of greatly reduced spontaneous bursting activity of neurons in the dorsal cochlear nucleus (DCN) in brain slices from Scn8a<sup>med−/−</sup>/Scn8amed−/− and Scn8amed−/+ mice. However, no hearing impairment has been previously reported for any Scn8a mouse mutants, and indeed hearing in dytogenic Scn8amed−/− mice (Scn8a<sup>1<sup>st</sup>/Scn8a<sup>1<sup>st</sup></sup>) was normal (Koay et al. 2002). Therefore, it is possible that the Cloth-ears mutation represents a unique gain-of-function allele in the auditory system. Thus, reassessment of other Scn8a alleles for subtle hearing deficits would be merited. Further historical and functional analysis of the auditory pathway in Scn8a<sup>Clth</sup> mice would identify which structures are primarily, and secondarily, affected. The Cloth-ears mutant is therefore the first evidence that mutation in Scn8a can result in hearing impairment in mice.

**Motor deficits in Scn8a<sup>Clth</sup>/Scn8a<sup>Clth</sup> mice**

Scn8a<sup>Clth</sup>/Scn8a<sup>Clth</sup> mice begin to display a continuous tremor at 8 days old, and later, show impairment at the wire manoeuvre test and the distal toe-pinch reflex, that suggests muscle weakness and distal sensory loss is occurring in these mice. The onset of skeletal muscle weakness or paralysis in the lethal Scn8a mutants is also from around 8 days old (Buchner et al. 2004; Duchen & Strich 1966; Lane 1976). Moreover, Garcia et al. (1998) showed that SCN8A was responsible for the threefold increase in sodium current between postnatal day 0 and day 8 in mouse motor neurons, and suggested that this was caused by postnatal upregulation of Scn8a. Thus, the onset of motor symptoms in Scn8a<sup>Clth</sup>/Scn8a<sup>Clth</sup> mice correlates with the reported upregulation of Scn8a in motor neurons. In contrast to almost all published Scn8a mouse mutants, we did not observe degenerative changes in skeletal muscle, cerebellum or any gait defects or paralysis in Scn8a<sup>Clth</sup>/Scn8a<sup>Clth</sup> mice. Particularly, this is markedly different to a non-lethal Scn8a mutant that shows a tremor: Scn8amed−/−/Scn8amed−/− mice have a marked loss of cerebellar Purkinje cells (Dick et al. 1985). A functional but mild...
impairment of one or more of these tissues could therefore underlie the motor deficits in Scn8a\textsubscript{Clth}/Scn8a\textsubscript{Clth} mice. Functional abnormalities previously identified in Scn8a mouse mutants include a loss of resurgent sodium current and disrupted firing patterns in cerebellar Purkinje cells (Raman et al. 1997), reduced conduction velocity and a prolonged refractory period in motor neurons (Duchen & Stefani 1971) and a failure of muscle fibres to show action potentials in response to nerve stimulation (Duchen & Stefani 1971). Electromyography/electrophysiological analysis of muscle, motor neurons and cerebellar output would be needed to delineate the origins of tremor and muscle weakness in Scn8a\textsubscript{Clth}/Scn8a\textsubscript{Clth} mice.

**Behavioural phenotypes in Scn8a\textsubscript{Clth}/Scn8a\textsubscript{Clth} mice**
Both Scn8a\textsubscript{Clth}/+ mice and Scn8a\textsubscript{Clth}/Scn8a\textsubscript{Clth} mice display an episodic tremor with behavioural arrest phenotype. This similarity of phenotype and its infrequent nature suggests that this is a dominant episodic phenotype occurring under particular external or internal conditions that could be seizure- or dystonia-like. Genetic and non-genetic factors are known to moderate some paroxysmal (episodic) neurological and motor disorders in mice and humans. For example, myotonia in the human disorder paramyotonia congenita caused by mutation in the SCN4A gene (OMIM: #168300) is aggravated and/or induced by exposure to cold temperatures (OMIM). Indeed, even Scn8a has recently been reported to modulate the severity of seizures in a mouse mutant of Scn2a (Martin et al. 2007). We tested a small cohort of Scn8a\textsubscript{Clth}/+ mice to see if handling or auditory testing induced tremor episodes, but only a small increase in tremor occurrence was observed (data not shown), indicating that these stimuli were not major inducers of tremor. Robust testing of the effect on tremor occurrence of other environmental factors such as temperature, and crossing to different genetic backgrounds, would be useful in isolating the precipitators of tremor episodes in these mice.

Alternatively, it could be that Scn8a\textsubscript{Clth}/+ and Scn8a\textsubscript{Clth}/ Scn8a\textsubscript{Clth} mice have heightened anxiety, leading to increased freezing (behavioural arrest). We found that the incidence and duration of freezing was significantly increased in Scn8a\textsubscript{Clth}/Scn8a\textsubscript{Clth} mice by visual observation. However, the decreased time of latency of first move into the light-half in the light-dark paradigm shown by Scn8a\textsubscript{Clth}/Scn8a\textsubscript{Clth} mice could suggest that these mice show lessened anxiety. Open-field analysis did not show any differences in locomotion and anxiety measures of Scn8a\textsubscript{Clth} mice. Furthermore, Scn8a\textsubscript{Clth}/Scn8a\textsubscript{Clth} mice displayed a greater frequency of trunk curling and limb grasping, which are often displayed by mouse mutants with neurological defects including Scn8amed\textsubscript{−/−} TgAA\textsubscript{Br} and Scn8amed\textsubscript{−/−} TgAA\textsubscript{Bs} mice (Kohrman et al. 1995). SCN8A is highly expressed in the hippocampus (Caldwell et al. 2000), which is involved in fear-related behavior. Recently, it has been reported that mice heterozygous for a null allele of Scn8a show evidence of ‘emotional’ behavioural defects, including increased freezing and avoidance of the centre in open-field tests (McKinney et al. 2008). This study indicates that, for some behavioural phenotypes, the null heterozygote is more severe than the Clooth-ears allele described here. In addition, the only family identified with a SCN8A mutation show neuropsychological impairment (Trudeau et al. 2006). A positive association between SCN8A variants and suicide attempts in humans has also been reported (Wasserman et al. 2005). It would be interesting to assess if a seizure-like or anxiety phenotype underlies the behavioural abnormalities in Scn8a\textsubscript{Clth} mice by electroencephalography and further behavioural testing.

**Mechanism of SCN8A dysfunction in Scn8a\textsubscript{Clth} mice**
Complementation testing showed that the Asp981Val mutation in SCN8A underlies the Scn8a\textsubscript{Clth} phenotype. Voltage-gated sodium channels are transmembrane proteins that allow fast influx of sodium ions into excitable cells in response to membrane depolarization, and are responsible, among other functions, for generating and conducting action potentials (reviewed in Ogata & Ohishi 2002). The widespread expression of Scn8a in the central and peripheral nervous system (Caldwell et al. 2000) and the critical function of SCN8A in generating sodium currents in neurons (Cummins et al. 2005; Garcia et al. 1998; Raman et al. 1997) suggest that functional defects in neuronal excitability could be responsible for the auditory defects, tremor and neurological/behavioural defects of Scn8a\textsubscript{Clth} mice. It is possible that the Asp981Val amino acid substitution causes a change in SCN8A channel kinetics or sodium current properties. Electrophysiology on another Scn8a mouse mutant with tremor [Scn8amed\textsubscript{−/−}/Scn8amed\textsubscript{−/−} (Ala1071Thr)] showed that the Scn8amed\textsubscript{−/−} mutation caused a shift in the voltage dependence of SCN8A channel activation (Kohrman et al. 1996), which could decrease neuronal excitability and thus result in ataxia and tremor. Additionally, the close proximity of the Asp981Val amino acid change to the predicted end of the D2-S6 segment (Fig. 1d) suggests that it is possible that this residue could be involved in the function of domain 2. Indeed, several mutations in the D2-S6 segment of the rat Scn2a have been shown to affect channel kinetics, including altering the voltage dependence of activation and inactivation, and causing incomplete inactivation (Yarov-Yarovoy et al. 2002). Also, we found that Scn8a\textsubscript{Clth}/Scn8a\textsubscript{Clth} mice showed abnormal dystonic postures after anaesthesia, that were not seen in Scn8a\textsubscript{Clth}/+ and wild-type mice. Interestingly, residues in the D2-S6 segment have been shown to be involved in the binding and action of anaesthetics to VGSCs (Kondratiev & Tomaselli 2003; Wang et al. 2001). Additionally, several amino acid mutations in other VGSC α-subunit genes that lie in very close proximity to the Asp981 amino acid have been identified in four human disorders. Functional consequences of these mutations include changes in fast inactivation and persistent currents (Green et al. 1998; Schwartz et al. 2000). Intriguingly, symptoms in these four human...
disorders are episodic, and believed or known to be precipitated by external or internal stimuli: the paroxysmal muscular disorders, paramyotonia congenita/myotonia congenita and myotonia fluctuans (SCN4A) (McClatchey et al. 1992; Ricker et al. 1994); near-sudden infant death syndrome (SIDS) caused by the cardiac condition long QT syndrome type 3 (LQT3) (SCN5A) (Schwartz et al. 2000) and severe myoclonic epilepsy of infancy (SMEI) (SCN1A) (Claes et al. 2001; Escayg et al. 2000). Thus, we predict that the Scn8a<sup>C<sub>th</sub></sup> mutant mouse could alter SCN8A channel kinetics or sodium current properties in Scn8a<sup>C<sub>th</sub></sup> neurons, which could be assessed in vitro and in vivo.

**The Scn8a<sup>C<sub>th</sub></sup> mouse is a resource for studying human disease**

The Scn8a<sup>C<sub>th</sub></sup> mouse is a novel mutant that links peripheral neural hearing loss with tremor and paroxysmal motor symptoms. A small number of mouse mutants with continuous tremor and hearing impairment have been reported (MGII, OMIM), including several other ion channel mutants [Atp2b2 (plasma membrane calcium-transporting ATPase 2): deafwaddler<sup>2J</sup> (Noben-Trauth et al. 1997) and deafwaddler<sup>2</sup> (McCullough & Tempel 2004); Spnb4 (spectrin beta 4): quivering (Parkinson et al. 2001); Kcnj10 (ATP-sensitive inward rectifier potassium channel 10) (Marcus et al. 2002, Neusch et al. 2001)]. However, no mutant mice with hearing loss and paroxysmal tremor have been reported. Around 12 human disorders feature both hearing impairment and tremor (OMIM). Essential tremor patients show significantly increased occurrence of high-frequency sensorineural hearing loss (Ono et al. 2003); however, a recent screen of patients with essential tremor did not detect any variants of SCN8A (Sharkey et al. 2008). Sensorineural deafness and tremor are also seen in the autosomal dominant demyelinating Charcot-Marie-Tooth disease type 1E (OMIM) and in 18q deletion dystonia syndrome (Gordon et al. 1995). The paroxysmal symptoms in Scn8a<sup>C<sub>th</sub></sup> mice and in human disorders caused by mutations in other SCN<sub>α</sub>-subunit genes suggest that mutation in SCN8A could also contribute to paroxysmal disorders in humans. It will be intriguing to consider SCN8A as a candidate gene for peripheral neural hearing loss and paroxysmal neurological or neuromuscular disorders in humans.

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Mutation in Scn8a in deafness and paroxysmal tremor

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Acknowledgments

We thank Rachel Hardisty-Hughes for critical review and helpful discussion, Abraham Acevedo-Arozena and Pat Nolan for critical review, Ian Russell for help and discussion of OAE data, Christopher Yau for statistical advice, Tim Randall and Lucie Vizor for mouse husbandry, Caroline Barker, Jennifer Corrigan and Adele Austin for expert histology and MRC Harwell core technical assistance.