***ABR Screening***

***WTSI recording protocols & analysis techniques***

ABRs were recorded in mice aged 14 weeks (± 3 days) using the methods described in detail in Ingham et al 2011. Mice were anaesthetized using Ketamine (100mg/kg, Ketaset, Fort Dodge Animal Health) and Xylazine (10mg/kg, Rompun, Bayer Animal Health) and placed on a heating blanket inside a sound attenuating booth. Sub-cutaneous needle electrodes were inserted in the skin on the vertex (active) and overlying the ventral region of the left (reference) and right (ground) bullae to record responses of the left ear.

Stimuli were presented as free-field sounds from a loudspeaker whose leading edge was 20cm in front of the mouse’s interaural axis. The sound delivery system was calibrated using an ACO Pacific 7017 microphone. Customized software produces an FFT-based (Fast Fourier Transformation) spectrum of the output of the loudspeaker across a wide range of frequencies. This curve is used to produce a correction (in dB) for each stimulus frequency used such that each test stimulus is presented at the required dB sound pressure level (SPL).

For threshold determination, customized software and Tucker Davis Technologies hardware were used to deliver click (0.01 ms duration) and tone pip (6, 18, 24, and 30 kHz of 5 ms duration, 1 ms rise/fall time) stimuli over a range of intensity levels from 10 – 95 dB SPL in 5 dB steps. Averaged responses to 256 stimuli, presented at 42.2/s, were analyzed and thresholds established as the lowest sound intensity giving a visually-detectable ABR response. (Following completion of recording, mice were passed on for DEXA/faxitron phenotyping assays and then recovered using Atipamezole; 1mg/kg, Antisedan, Pfizer)

For each mouse, once installed in the recording apparatus, a fixed recording protocol is followed :

***Initial test ABR.*** An ABR to 70dB click stimuli is recorded. This allows a visual check of the electrode trace to ensure a relatively low noise recording environment is achieved and also provides a reference response amplitude for QC checking. At this stage, electrode positioning can be adjusted to give a better signal : noise ratio if needed. This ABR can also give an insight as to whether a mouse may have a hearing impairment (eg. if the ABR amplitude is particularly small) or of there may be abnormalities in waveform shape (an experience user can indentify significantly abnormal ABR waveforms with relative ease).

***Estimation of Heart Rate.***A single trace of 5s duration, using a single 0dB SPL 42kHz tone-pip, is recorded, to capture the ECG waveform overlying the evoked potential trace. Counts of ECG peaks are used to estimate the heart rate of the mouse before threshold recording begins. ECG components of the each recorded trace are minimized, along with electrical noise, during the averaging process when recording ABRs.

***Determine click-evoked ABR threshold.*** A series of click-evoked ABRs are recorded, ranging from 10-85dB SPL in 5dB intervals and used to determine click threshold.

***Determine tone-evoked ABR thresholds.*** Tone-evoked ABRs are recorded for a fixed set of frequencies (6, 12, 18, 24 & 32 kHz) over sound levels ranging from 0 – 85dB SPL in 5db intervals. For routine screening, different SPL ranges are recorded for different test frequencies to improve time efficiency (6 kHz, 20 to 85 dB; 12 kHz, 0 to 70 dB; 18 kHz, 0 to 70 dB; 24 kHz, 10 to 70 dB; 30 kHz, 20 to 85 dB). Responses are recorded in an array beginning the lowest stimulus level, in decreasing frequency order before stepping up to the next (5dB higher) stimulus level. These responses are used to determine the ABR thresholds to the tone stimuli. If mice appear to have hearing impairment, the upper limit of SPLs is extended to 95 dB for each test frequency and for clicks (this represents the upper limit of the linear range of our sound system at these frequencies).

***Estimate Heart Rate.*** A second estimation of heart rate is made, for comparison with that taken earlier in the screen.

***Final test ABR.*** Another ABR to 70dB click stimuli is recorded, for comparison with that taken at the beginning of the screen.

For the ABR screen, we aim to test a minimum of 4 mutant mice per line (of either sex). For other tests on the pipeline, 14 mutant mice (7 males & 7 females) are required. Phenotyping cohorts are issued when mice are available, such that several partial cohorts are issued to achieve the required number of mice for a single line. This allows the ABR assay to pick up further mice from any lines which exhibit any features of interest (see later) to extend the number tested beyond the target of 4. In addition to mutant mice, at least 4 wildtype mice are tested each week, from each core genetic background of the mutants tested each week. These wildtype results form a local control group for comparison with the mutant lines and also contribute to a large reference range of control data which is used to determine is ABR results from a mutant line are of interest.

**ABR Screening Data Analysis**

Each mouse tested has a unique identifier (at the WTSI, “Mxxxxxxxx”, where “xxxxxxxx” is an 8-digit number allocated sequentially to each mouse born in the animal facility). This unique identifier forms the filename of the ABR data recorded, which the WTSI Averager software saves in “.csv” format, for example “M00363888.csv”. (An annotation of the structure of the raw data files can be found in the accompanying spreadsheet “DataFile\_Annotation.xls”). These files contain metadata for the mouse and experiment, calibration / equalization data, along with information detailing all stimuli used and all evoked potential trace data recorded.

Each datafile is uploaded to the WTSI Mouse Tracking Database (a customized in-house database application). Within this database, ABR traces recorded for each mouse are displayed for thresholds allocation, wildtype threshold reference range data are calculated, mutant mouse population threshold results are displayed along with wildtype threshold averages, reference ranges, etc. Currently, further analyses extending beyond allocation of threshold are performed offline outside of the WTSI mouse tracking database.

***ABR Threshold & Hearing Sensitivity.***

For each stimulus used (click and 5 tone frequencies), ABRs recorded over the range of sound levels tested are plotted as a stack, ordered by increasing dB SPL. Threshold is estimated by visual inspection of the stacked ABR traces as the lowest sound level where any component of the ABR waveform (for example, a positive or negative waveform) is recognizable and can be traced up to higher stimulus levels. We decided against the use of automated algorithms to determine threshold in favour of using a skilled scientific eye. Click threshold is plotted along with an audiogram made of up of thresholds of the frequencies tested to give a profile of the hearing sensitivity of each mouse.

***Waveform Shape Comparisons.***

Plots are generated from click-evoked ABR waveforms of mutant & local control mice, where waveforms recorded at 20dB sensation level (SL, dB above threshold) and 50dB SL are overlaid. We also plot an average (with standard deviation range) of the waveforms to facilitate comparisons of mutant to controls. This simple comparison can be enough to detect gross abnormalities of the waveforms and identify potential phenotypes in mutant lines. Currently, these waveform comparisons are not performed within the WTSI mouse tracking database. We use an accessory piece of WTSI software (Traceview) to select appropriate traces and copy the waveform data to spreadsheets for grouping, analysis and display of waveforms of multiple control and mutant mice.

In some cases, it is possible to identify mutant lines such gross waveform abnormalities at the time of data collection. In addition to this simple waveform comparison we perform a quantitative analysis of various parameters associated with the waveform which can be used to support the qualitative observations of waveform shape and even to detect more subtle differences that may not be obvious in visual inspection of the waveforms.

***Input/Output Functions (IOFs).***

Using click-evoked ABRs, waveforms are analyzed in detail, to determine the amplitude and latency of the first and third positive (P1 and P3) and negative peaks (N1 and N3) of the waveform at each stimulus level recorded. This is performed using software routines developed by Brad Buran and kindly donated for our use by M.C. Liberman (Harvard University). In our recording equipment and using these particular genetic background strains, peak I and III are most easily identifiable across sound levels. From these parameters, we calculate the peak-peak amplitude of waves I & III and the interval from positive peak I to positive peak III and the interval from negative peak I to negative peak III.

Datafiles are loaded into Traceview, click-evoked ABRs selected and their waveforms exported in a format compatible with Buran’s ABR Peak Analysis program. The export file is a tab-delimited txt file named according to the following convention – ABR datafile name, suffixed by “\_Click\_for ABR Notebook.txt”; for example “M00363888.csv\_Click\_for ABR Notebook.txt”. (An annotation of the structure of these files can be found in the accompanying spreadsheet “Waveforms\_Annotation.xls”)

When opened in Buran’s ABR Peak Analysis software, these traces can be analyzed and the latency and amplitude of up to 5 positive and negative peaks determined. We use markers 1 & 2 to determine the peaks of waves 1 and 3. Once confirmed by eye that the correct peaks are allocated, the latency and amplitude information can be saved and exported in a tab-delimited txt file, with the suffix “-analyzed.txt” added to the name of the imported file; for example “M00363888.csv\_for ABR Notebook.txt-analyzed.txt”. (An annotation of the structure of these files can be found in the accompanying spreadsheet “IOF\_Annotation.xls”)

Several types of Input-Output Functions are then plotted. We examine how the peak-peak amplitude of Wave I and III, latency of P1, N1, P3 & N3 and the P1-P3 and N1-N3 interval vary with sound level. IOF curves are plotted relative to click threshold for each mouse (ie parameter plotted against dB sensation level). As these parameters clearly vary with sound level, it is important to plot IOFs relative to click threshold to reduce variability in the parameters caused by variations in click threshold between individual mice. IOFs of individual mice (mutants and local controls) are plotted over a reference range generated for each parameter. The IOF data for mutants and local controls are handled in Excel spreadsheet analysis templates. Wildtype data are compiled together in Excel to produce median and reference range baseline data. Individual IOFs for mutants and local control are then plotted overlying the reference range.

**Quality Control.**

***Visual inspection*** of the ABR traces recorded is used to look for significant noise or artifact on the recordings which is accounted for when allocating threshold & other parameters. For example, excessive myogenic noise on the recording can effectively mask an evoked potential, elevating the threshold estimate. On rare occasions, the ECG component become partially synchronised with the duty cycle of the trace recording and averaging to give a large partially averaged ECG artifact which again may mask the ABR component if overlapping in time.

***Allocation of Threshold.*** Thresholds are allocated by experienced experimenters using the criteria outlined above. Data from random mice are viewed by a second experimenter and the estimate of threshold confirmed.

***Click “start” v Click “end” ABRs.***  The peak-peak amplitude of click-evoked ABR wave I & wave III is compared at the end of testing relative to that recorded at the start. Based on a population of 1000 normal hearing mice, a reference range of % amplitude change was determined. Any mice where this comparison produced a reduced % for either wave I or wave III, below the 1st percentile of the reference range, was excluded from further analyses on the assumption that there may have been some physiological deterioration in the mouse during the ABR recording period.

**Heart Rate.** The estimated heart rate at the start and the end of testing are compared as a % of the starting rate. Based on a population of 500 normal hearing mice, a reference range of % change in heart rate was determined. Any mice showing falls in heart rate below the 1st percentile of the reference range may be compromised physiologically and are excluded from further analysis.

**Significant Calls on Mutant Data**

We apply similar rules used by the WTSI MGP to make calls on mutant datasets, rather than more traditional statistical tests (eg. t-tests, or analysis of variance).

***ABR thresholds.*** Data for mutant lines are considered significant and of interest if there are at least 4 genotype-locked mice and that over 60% of observations fall outside the reference range for the click or any of the 5 frequency stimuli.

***Waveform Shape.*** For mutant lines showing abnormal ABR waveform shapes, the criteria are somewhat more subjective in definition. It may that the waveform shape change is subtle such that it is detected as a change in the IOF for amplitude, latency or interval. Alternatively, the waveform may be perturbed to such an extent that accurate identification of peaks (and therefore, IOF analysis) is not possible.

***Input-Output functions.*** A mutant line is considered significant if there are at least 4 genotype-locked mice and that over 60% of observations for a particular parameter fall outside of the reference range for at least 5 adjacent dB sound levels (ie a 20dB range).