SANGER INSTITUTE
STANDARD OPERATING PROCEDURE

SUBJECT: Auditory Brainstem Response (ABR) – V1

SOP Number: SOP0100	**To be reviewed:**	
Author(s):	Signed:	Date:
Editor:	Signed:	Date:
Risk Assessor:	Signed:	Date:
Date of Implementation:		

INTRODUCTION:

The purpose of this procedure is to assess hearing in anaesthetised mice by determining the thresholds of the ABR in wild-type and genetically altered mice.

ABBREVIATIONS:

ABR = Auditory Brainstem Response
dB SPL = Decibels Sound Pressure Level
DCF = Data Capture Form
DEXA = Dual Energy X-ray Absorptiometry
ECG = Electrocardiogram
IVC = Individually Ventilated Cage
KX= Ketamine Hydrochloride/Xylazine Hydrochloride
LAA = Laboratory Animal Allergens
PAF = Project Authorisation Form
PIL = Procedure Individual License
PPE = Personal Protective Equipment
PPL = Procedure Project Licence
QC = Quality Control
RA = Risk Assessment
RSF = Research Support Facility
SMP = Sick Mouse Procedure
SOP = Standard Operating Procedure

QUALITY CONTROL (QC) DURING PROCEDURE:

Refer to the table below for approved QC fail comments steps to be used during procedures.
If a value has been collected leave on the Data Capture Form (DCF) and then apply the fail reason from below;

In-Life Procedures:

Problem / Issue	QC fail reason
At any point during the procedure the mouse is deemed sick and processed through Sick Mouse Procedure (SMP)	Fail whole DCF as 'Sick mouse' – for all tests that day
Mouse incorrectly scheduled at wrong week	Fail whole DCF as 'Scheduling Issue'
Insufficient anaesthesia level affects the whole test DCF	Fail whole DCF as 'Anaesthesia Issue'
Insufficient anaesthesia level affects specific parameter(s)	Fail parameter(s) as 'Anaesthesia issue'
A welfare issue makes it impossible to collect specific parameters	Fail parameter(s) as 'Welfare issue'
Parameters affected by delays or noise due to fire alarms	Fail parameter(s) as 'Fire alarm'
An equipment failure affecting specific parameters	Fail parameter(s) as 'Equipment failure'
A software issue affecting specific parameters	Fail parameter(s) as 'Software failure'
A procedural error which affects data collection	Fail parameter(s) as 'Manual error'
Parameter cannot be assessed	Fail parameter(s) as 'Readout not possible'
Wrong value has been entered which cannot be re-measured or accounted for	Fail parameter(s) as 'Erroneous data'
Glucose meter unable to record high blood values	Fail parameter(s) as 'Meter reading HI'
Fighting occurs prior to or during data collection	Fail parameter(s) as 'Fighting during procedure'
Parameter on the current DCF is not required for that specific test/pipeline	Fail parameter(s) as 'Not required'

HEALTH & SAFETY:

This procedure is covered by the following Risk Assessment (RA):

Name: WTSI-1498
Assessment Title: Auditory Brainstem Response
Assessor:
Approver:

- Appropriate Personal Protective Equipment (PPE) is to be worn at all times when handling animals. This includes:
  - Overshoes
  - Gown
  - Gloves
- In addition to the above, when sources for Laboratory Animal Allergens (LAA) (animals or soiled cages) are not contained within Local Exhaust Ventilation Systems (change stations, fume hoods or down flow tables), a respiratory mask, for which you have passed a face fit test, must be worn.
- Lone worker alarms should be used when working alone.
- This procedure can only be performed during Research Support Facility (RSF) core hours (7:30am-7:30pm).

- All electrical equipment is to be inspected for damage before use.

RESPONSIBILITIES:

All staff performing this procedure are responsible for ensuring that this Standard Operating Procedure (SOP) and accompanying Risk Assessment have been read, understood and where applicable is followed in accordance with the relevant Procedure Project License (PPL). All staff should be trained and competent to perform the procedure, where applicable they should also be licensed to perform the procedure with a valid Procedure Individual License (PIL).

For secondary phenotyping, seek confirmation with project manager for deviations from this SOP. Any deviation will be detailed in the Project Authorisation Form (PAF).

RESOURCES:

Equipment:

1. Balance
2. 70% Ethanol and tissues – **Hazardous substance: highly flammable**
3. Hydrex Pink Hand spray **- Hazardous substance: highly flammable**
4. Hydrex Hard Surface spray - **Hazardous substance: highly flammable**
5. One clean cage and 2 nestlets per cage of mice tested
6. Heat mat
7. 1 x 1mL BD Plastipak syringes
8. BD Microlance 3, ½"G needles; one for each mouse – **Sharps hazard: needles must not be re-sheathed once opened, dispose into sharps bin as soon as finished.**
9. Yellow sharps container
10. 100mg/kg Ketamine Hydrochloride,10mg/kg Xylazine Hydrochloride (K/X) solution (anaesthetic)
11. 1mg/kg Atipamezole hydrochloride (Antisedan) solution (reversal)
12. Viscotears (liquid eye gel containing 2 mg/g Carbomer, with cetrimide; *Supplier name; Dr Mann-Pharma*)
13. Post-anaesthetic care checklist
14. Anaesthesia cage labels
15. Absorptiometry & Imaging Record Sheet
16. ABR mouse labels
17. Tecniplast heated Individually Ventilated Cage (IVC) recovery rack
18. Countdown timer with alarm (*Supplier name; VWR International Ltd. Supplier product code; 609-0131)*
19. Timer
20. 110mg/Kg Ketamine; 110mg/Kg Xylazine dose calculation chart
21. Sound-attenuating chamber (*Supplier name; Industrial Acoustics*)
22. Heating blanket
23. TDT System 3 to record ABRs
24. Digital Oscilloscope
25. Needle electrodes (*Supplier name; UniMed; Supplier product code; SD51-426-V1*) - **Sharps hazard: use needle holders to insert electrodes, dispose into sharps bin when blunt.**
26. "Phenotyping in Progress" sign
27. Post Procedure Check labels

Associated SOPs/Documentation:

- **SOP0023** – Anaesthesia of Mice with Avertin for X-Rays
- **SOP0024** – Anaesthesia of Mice with Ketamine-Xylazine + Antisedan for X-Rays
- **SOP0031** – Recovery of Mice from Anaesthesia
- **SOP0032** – Preparation of Ketamine-Xylazine + Antisedan
- **SOP0045** – Weigh Mice
- **SOP0054** – Dual Energy X-ray Absorptiometry and X-ray Imaging
- **SOP0101** – Taking and Returning Cages for Procedures
- **SOP0146** – Initiation of Welfare Assessments and Sick Mouse Procedure
- Ketamine_Xylazine_Antisedan calculation template
- Anaesthesia Recovery Log Sheet_main
- Anaesthesia cards TEMPLATE
- ABR mouse label
- 110% calculation chart
- ABR Troubleshooting Guide

Staff: This test can be completed by one phenotyper.

NOTE:

On some pipelines, the ABR procedure was performed along with SOP0054 - Dual Energy X-ray Absorptiometry and X-ray Imaging, in this workflow mice were anaesthetised and reversed by the phenotyper performing SOP0054.

Initially ABR was performed with 3dB increments, but this was later switched to 5dB increments. The anaesthesia used was both pipeline and welfare dependent.

PROCEDURE:

Before performing any tests verify this is the correct set of procedures at this time point in the pipeline or project, by consulting the cage card(s).

1. Preparation

1.1. Collect scheduled mice from the animal room, transport them to the procedure room and register them to the correct rack (Refer to SOP0101 – Taking and Returning Cages for Procedures).
1.2. Place ‘Phenotyping in Progress’ sign on the outside of the door.
1.3. Turn on heat mat, bring the anaesthetic and reversal solutions (pipeline dependent) up to room temperature. Ensure all electrical equipment is switched on at least 10 minutes prior to start, to allow microphone amplifier / heating blanket etc. to reach operating conditions.
1.3. Log on to the computer.

2. Calibrate sound system and software

2.1 Open **Averager** ABR recording software by double-clicking on the **Averager** shortcut on the desktop.
2.2 On the **Animal and Experiment Information** window (see Fig. 1), type in today’s date (in the format YYYY_MM_DD) in the **Mouse ID** field.
2.3 Click **Proceed**.

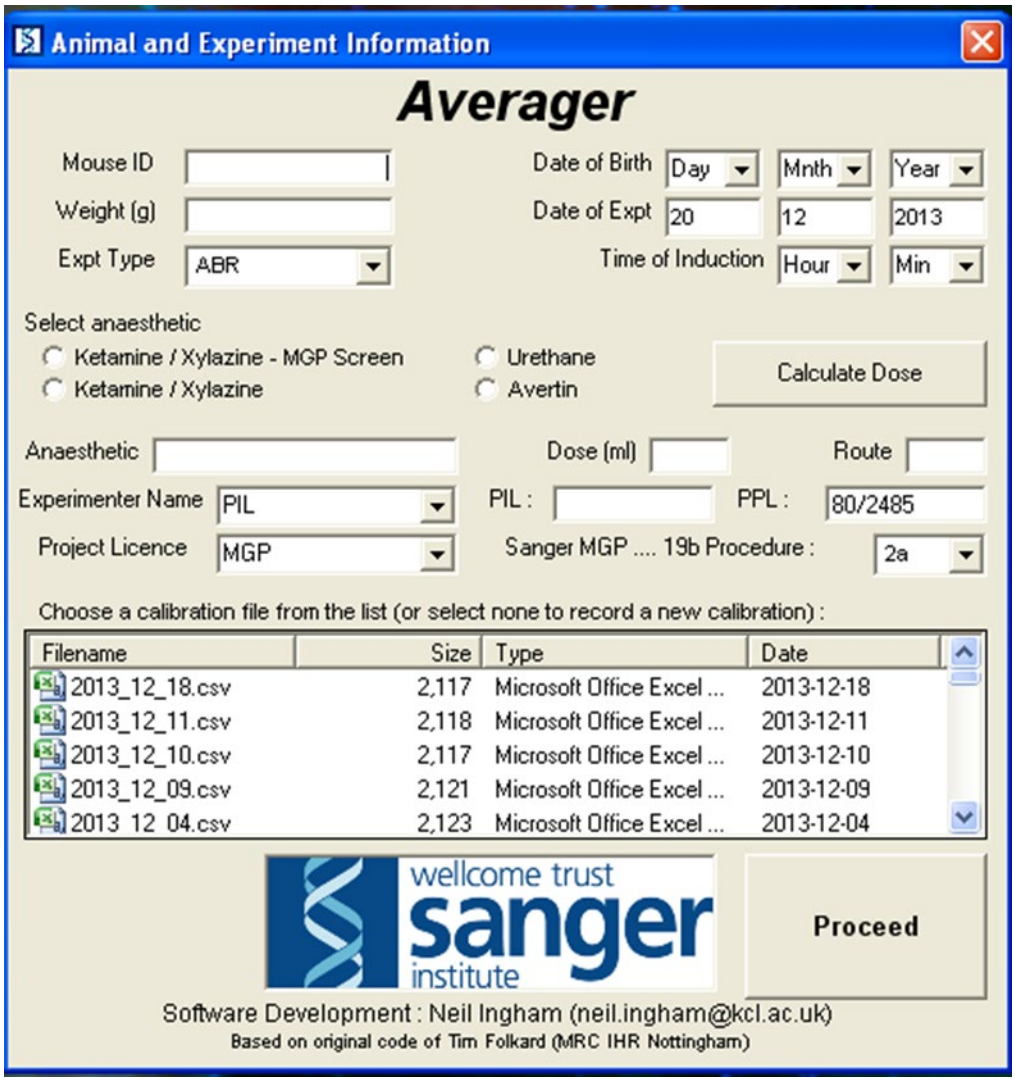

Fig. 1. Showing the fields to be completed on the Animal and Experiment Information screen for Averager.

2.4 Place the microphone in position for calibration in the sound attenuating box, aligning it with the lines marking the placement of the mouse's interaural axis and slightly to the left of the mouse's midline, and close the door.

2.5 Click on **File** in the top left hand corner of Averager to open a drop-down menu, click **Load Setup** to open up a second drop-down menu. Under the **Calibration** heading, click on **Calibration by White Noise**. Make sure that the doors to the ABR room are shut or the calibration will be wrong.

2.6 Ensure the **Save Calibration to File** box is ticked.

2.7 Click **Run**.

2.8 When recording of the calibration is complete, a box will prompt the user to enter calibration filename.

2.8.1 If the calibration curve appears correct, enter this as today's date (in the format YYYY_MM_DD) and press **OK**.

2.8.2 **If any of the doors in the ABR room are opened during the calibration, the calibration will be distorted.** If this happens press **No** and perform the calibration again by clicking on **Run.**

2.9 Turn off the microphone.

3. Mouse Preparation for Recording

3.1. In addition to disposable gloves, gown and overshoes, **a face mask which has been fit tested must also be worn when mice are out of containment**

3.2. Fill out an ABR Mouse Label for each mouse to be tested with:

3.2.1. Mouse barcode
3.2.2. Mouse ID
3.2.3. Weight
3.2.4. Earmark
3.2.5. Sex

3.3. Anaesthetise mice according to the appropriate anaesthesia SOP. If ABR is done as part of the workflow with DEXA and faxitron, the phenotyper performing DEXA will anaesthetise all of the mice.

3.4. Open **Averager** and on the **Animal and Experiment Information** window, fill in the following:

3.4.1. Type in the mouse barcode in the **Mouse ID** field.
3.4.2. Type in mouse weight in the **Weight (g)** field.
3.4.3. Type in the data of birth in the **Date of Birth** field.
3.4.4. From the drop down in the **Time of Injection** field enter the time that the mouse was injected.
3.4.5. Select the appropriate anaesthetic from the **Select anaesthetic** section.
3.4.6. Click on the **calculate anaesthetic dose** button – this calculates the required dose and autocompletes the appropriate anaesthetic route.
3.4.7. Select the experimenter's name from the **Experimenter Name** drop down. This autocompletes the **Experimenter PIL** field.
3.4.8. The correct project license number is already in the **PPL** field. Mild procedure is selected as default in the drop down; change if mice are tested as a moderate procedure.
3.4.9. Select the calibration file for the day.
3.4.10. Click **Proceed**.

3.5. Once anaesthesia has been reached, place the mouse on the heating blanket in the sound attenuating chamber.

3.6. Insert needle electrodes **using needle holders to do so**:

3.6.1. Insert the **ground** (gnd) electrode – green or white – through the skin overlying the right bulla.
3.6.2. Insert the **reference** (ref) electrode – blue or black – through the skin overlying the left bulla
3.6.3. Insert the **active** electrode – red or yellow – through the skin on the vertex (See Fig. 2).

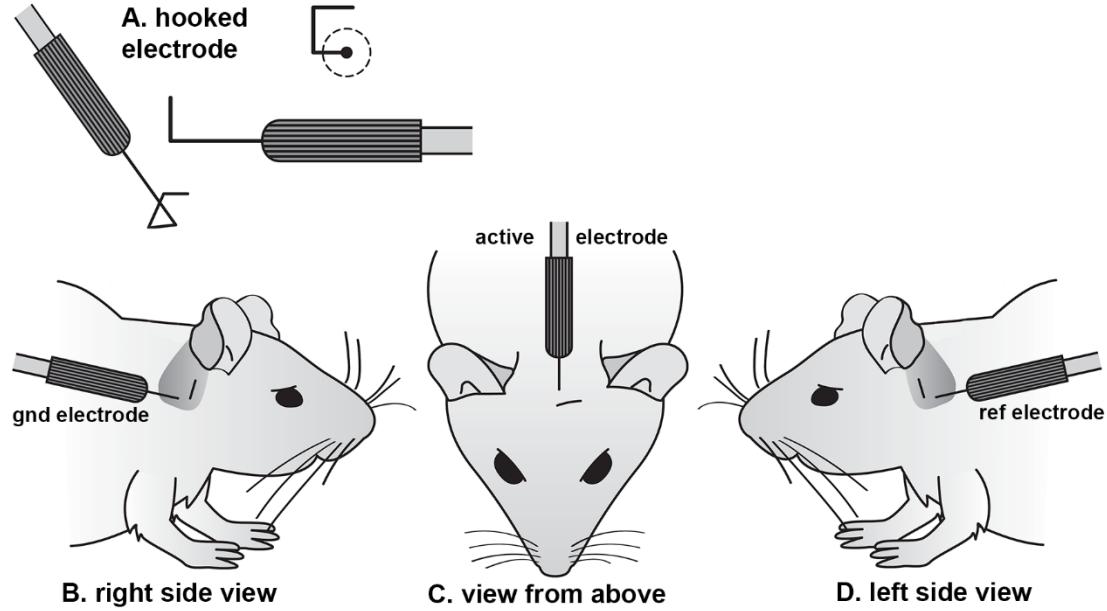

Fig. 2. Position of sub-dermal needle electrodes for ABR recording.

3.7. Place the mouse ventrally on the heated mat, with its head pointing towards the speaker. Ensure its nose and mouth are not obstructed. Place the mouse so that the leading edge of the speaker is 20cm from the level of the mouse's ears (interaural axis). See Fig. 3. There are lines marking the placement of the mouse's interaural axis and the mouse's midline on the plastic covering of the heated blanket.

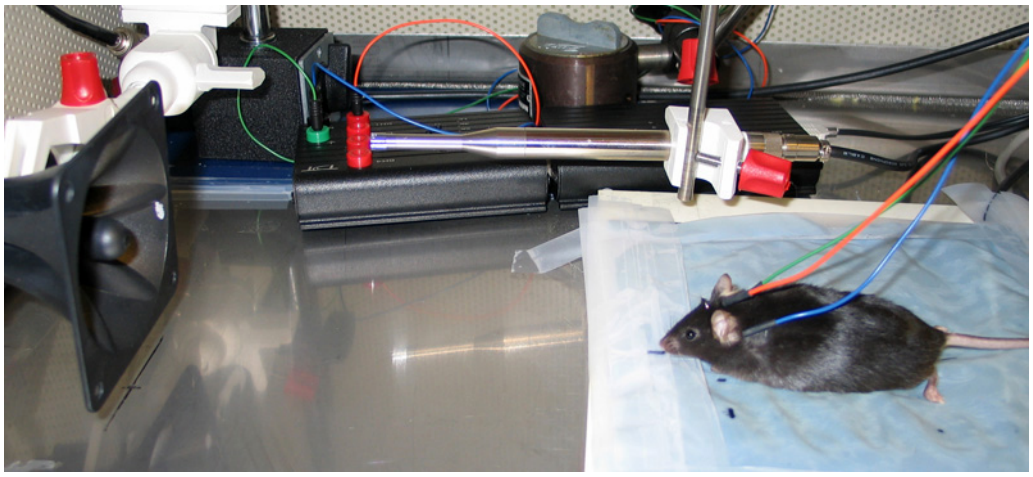

Fig. 3. Position of mouse directly in front of the sound source.

3.6 Apply one drop of viscotears to each eye.

3.7 The oscilloscope should give a relatively low-noise trace (2-5V peak) with a clear Electrocardiogram (ECG) visible. If it is not, remove one or more electrode and reinsert into the correct position.

4. Auditory Brainstem Response Recording

4.1. In the **Averager** software, record the first **Click ABR** to confirm recording apparatus is functioning (see Fig. 4).

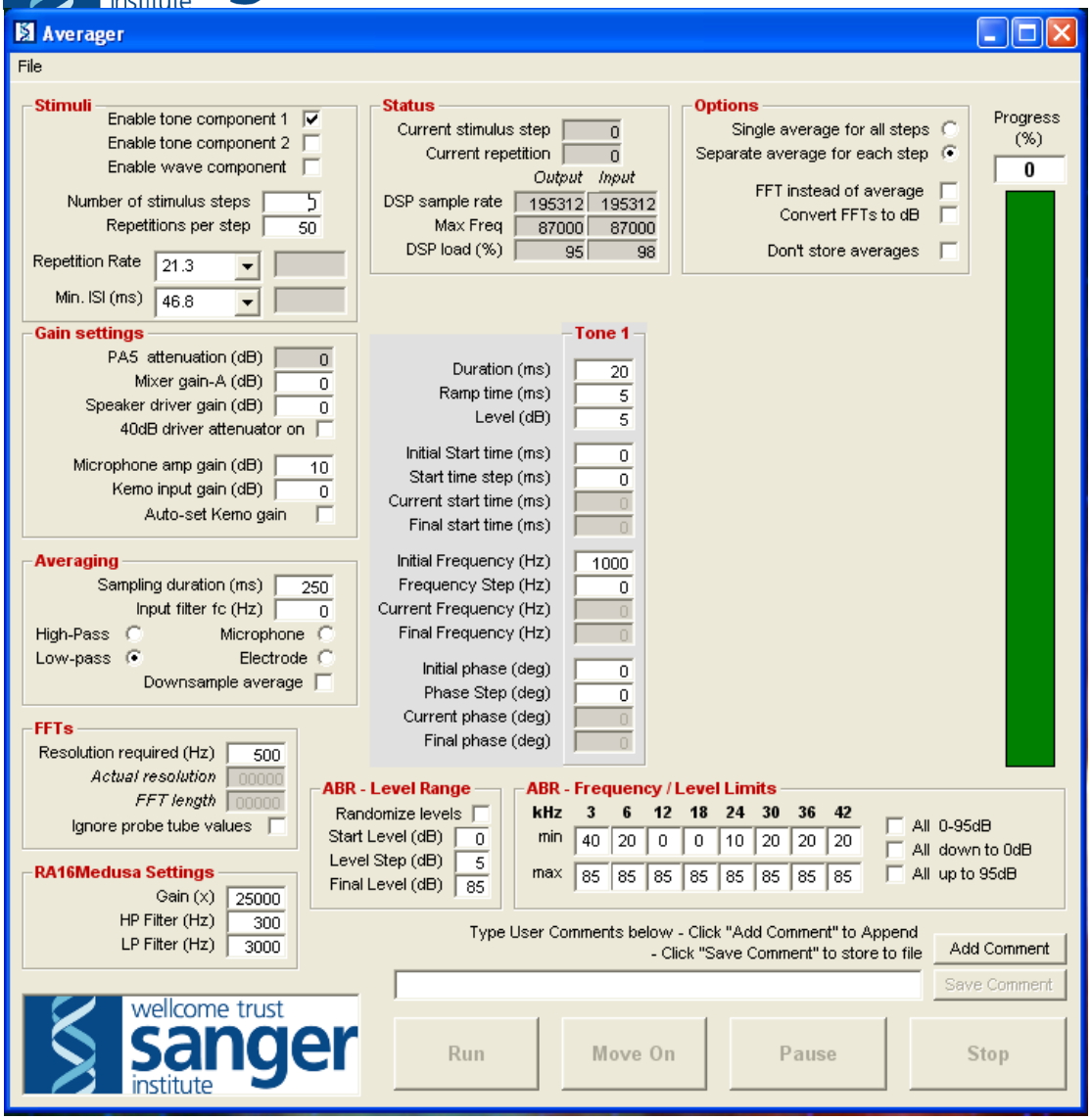

Fig. 4. Showing the Averager interface screen. All parts of the ABR test are controlled from this screen.

4.1.1. Click on **File** in the top left hand corner of the screen, click on **Load Setup** in the drop-down menu, this opens up a second drop-down menu, under the **Auditory Brainstem Response** heading select **Click ABR** (see Fig. 5).

4.1.2. Click **Run** to present a test click stimuli (click @ 70dB SPL). From this it is possible to see whether a clear ABR can be recorded.

4.1.3. If the signal is small or the recording appears noisy on the oscilloscope, adjust the position of one or more of the electrodes.

4.1.4. If no ABR can be seen:

4.1.4.1. Check the placement of the electrodes in the scalp, try removing them and re-inserting them.

4.1.4.2. Check that the speaker is producing a sound; it should be possible to hear the **Click ABR** with the door to the soundproof box open.

4.1.4.3. The mouse may have a hearing impairment, in which case there will be no ABR. Tick the box that says **All up to 95 dB** in the **ABR – Frequency /Level Limits** box.

4.1.4.4. If there is no heart beat and no ABR present, check that the mouse is still alive. If the mouse has died, tell the x-ray team, and refer to SOP0146 – Initiation of Welfare Assessments and Sick Mouse Procedure.

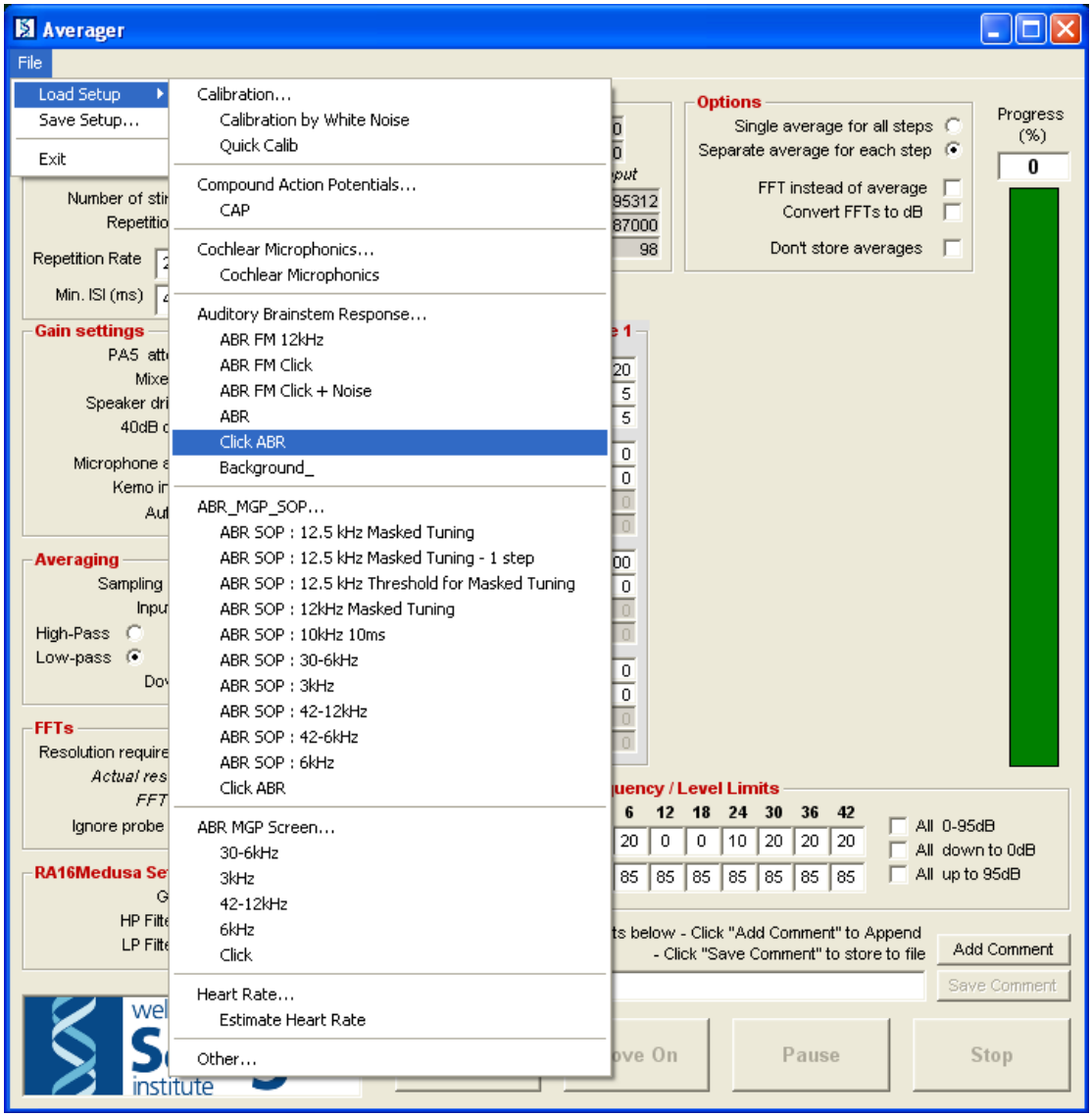

Fig. 5. How to select Click ABR from the Averager drop-down menu. All stimulus options are selected this way.

4.2 Record an ECG trace to determine Heart Rate.

4.2.1 Click on **File** in the top left hand corner, from the drop-down menu select **Estimate Heart Rate** in the **Heart Rate** section at the bottom of the menu.

4.2.2 Click **Run** to record a single sweep to display the ECG. Start the timer.

4.3 Record Click-Evoked ABRs for determination of click threshold.

4.3.1 Click **File** to open the drop-down menu, from the **Load Setup** menu**,** under the **ABR MGP Screen** heading, select **Click.**

4.3.2 Click **View on Monitor 1**.

4.3.3 The data collected will be displayed on-the-fly in the newly opened array to aid immediate visual interpretation of results. In addition, responses are displayed one level at a time in the stimulus window.

4.3.4 Click **Run**.
4.3.5 ABR recordings will be made using an array of click stimuli from 0 - 85dB SPL in 3 or 5dB steps (pipeline dependent).
4.3.6 The data collected will be displayed on-the-fly.
4.3.7 If mice show high ABR thresholds, tick the box that says **All up to 95dB**.

5. Record Tone-evoked ABRs for determination of tone thresholds.
5.1. Select **File > Load Setup > ABR MGP Screen >30-6 kHz.**
5.2. Click on **Run**.
5.3. ABR recordings are made using an array of stimuli (frequencies and levels). The run will present tone pips across 5 frequencies (30, 24, 18, 12, 6 kHz) at intensity levels defined in the **ABR – Frequency / Level Limits** box. Usually these extend to 85dB SPL except in recordings where **All up to 95dB** has been selected.
5.4. The **Pause** button can used to temporarily halt recording should the need arise (after the current stimulus has finished), e.g. to check the condition / status of the mouse, to give anaesthetic top-up dose, etc.
5.5. If, upon concluding the recording of the ABR for the tone pips, clear ABRs cannot be seen at one or more frequencies, even at the highest sound level, you can record a few extra levels by adjusting the **ABR – Level Range.** Change the **Start Level** to 90 and the **Final Level** to 95. In the **ABR – Frequency / Level Limits** box, change the **min** to 90 and the **max** to 95 for any frequency where you wish to record additional sound levels.

6. **Normal workflow** – used when x-ray test one mouse for each mouse undergoing ABR. This is typically 13-15 minutes per mouse
6.1. For the normal workflow, give a call to the x-ray operators when the timer reaches 5 minutes 30 seconds.

7. Record an ECG trace to determine Heart Rate.
7.1. Select **Estimate Heart Rate** from the drop-down menu.
7.2. Click **Run** to record a single sweep to display the ECG.

8. Record a final Click ABR to complete the recording
8.1. Select **Click ABR** under the **Auditory Brainstem Response** heading in the **Load Setup** drop-down.
8.2. Click **Run** to present 70dB SPL test stimuli to record the final test ABR.

9. Uploading of data & transfer of mice
9.1. When recording from an animal is complete, upload the ABR data to the database.
9.2. If a mouse has been injected for ABR, but does not undergo ABR recording, or it is not possible to upload the data file for any reason, the ABR procedure must still be recorded on the database.
9.3. Carefully remove the recording electrodes from the mouse and remove the mouse from the sound attenuating box.
9.4. Pass the mouse along with the ABR Mouse Label to the x-ray operators for continued testing (pipeline dependent) and move to step 11. Otherwise move to step 10.

10. **Recovery of anaesthetised mice which are not undergoing X-ray procedures.** Monitoring of anaesthetised mice is necessary during this procedure. Initiate recovery - refer to SOP0031 - Recovery of Mice from Anaesthesia.

All cages must display the updated cage card. Place a 'POST PROCEDURE CHECK REQUIRED' label on all cages and register them to the correct rack whilst returning them to their destination/home rack in the animal room (Refer to SOP0101 – Taking and Returning Cages for Procedures).

11. Finishing after the ABR experiment

11.1. When all the mice have been tested, turn off the oscilloscope, lamp, Medusa headstage, heating blanket and TDT equipment.

11.2. Clean all equipment, surfaces and the floor. **Transfer all waste to a yellow clinical waste bag or clearly labelled waste container. This is transferred to blue suite for disposal as appropriate.** Take care not to touch the microphone with anything damp as this may cause damage.

12. **Exporting data for Input-Output data analysis**. This is used in the third phase of data analysis.

12.1. Open up **Traceview**, click on **File** in the top left hand corner of the screen select **Load** from the drop-down menu, select the **data** folder from the computer's local C: drive, type in the mouse barcode.

12.2. Open the file, select the check boxes at 10 and 85dB SPL (or 95dB SPL if applicable) traces and click **Trace** in the top left hand corner and click **Select block** in the drop-down menu – this will select all the traces in the click stack between 10 and 85dB SPL (or 95 if applicable).

12.3. Select **Plot** in the top left hand corner, click on **Overlay selected traces** in the drop-down menu, then click **Plot** and select **Export selected traces for waveform analysis** from the drop-down menu.

13. Saving data to the team drive

13.1. At the end of a day's experiments, copy all the ABR data (including the analysed files) from the local C: drive to the appropriate drive.

13.2. Electronic records are kept for each day of testing, this allows notes to be made on specific lines or mice regarding anything of interest during ABR testing, and also allows recording of incidents during ABR e.g. electrode breakage, raised thresholds, extending protocol to 95dB SPL, excessive noise during recording.

TROUBLESHOOTING:

No signal on the oscilloscope:
Ensure all equipment is switched on.
Make sure Averager is running. Sometimes you may not see a signal without Averager switched on.
Check that the headstage and the preamp are properly connected – to check this gently pull them apart and push them back together. If this is the source of the problem, a signal should appear on the oscilloscope.

Mouse not sufficiently anaesthetised:
Check the appropriate anaesthesia SOP to see if a top-up is appropriate and the required volume. If a top-up is given, wait a few minutes and see if the mouse is sufficiently anaesthetised. If no top-up is appropriate or even with one the mouse is insufficiently anaesthetised for ABR recordings, pass the mouse to the x-ray team. Make a note of any top-up dose and time if appropriate.

Error message when trying to start up Averager:
'RP2(1) not running' means the TDT equipment isn't on. Make sure all the equipment is turned on, run Averager as normal. The rechargeable headstage battery may be flat if it hasn't been charged.

When running the click stack, all the ABR responses look the same:
The attenuator has not reset. Stop the recording. There are two options:

1) Delete the file, restart Averager and re-enter the mouse data.
2) Close and reopen Averager and re-enter the mouse data. When the dialog box warns you that there is already a file with that name and that the new file will over-write it, click OK.

If you notice the attenuator has not reset when you open Averager but before the Calibration is run – DO NOT close Averager. Run the Calibration, then close Averager and follow the instructions above for re-starting a recording.

Wrong mouse anaesthetised or mislabelled ABR file:
If you realise that the mouse that has been anaesthetised is the wrong mouse:

- Firstly, tell the x-ray team and find out which mouse is being tested next.
- If the mouse is correctly identified before ABR recordings have been started, enter the correct information into Averager and correct the information in the lab book.
- If the information for the mouse has already been put into Averager and recording has started, finish the recording and email the affected file to the person responsible for ABR so that it can be corrected prior to uploading to the database.
- If necessary, add the ABR procedure through the 'view cage' page, so that the ABR procedure appears on the database and the cage card.

Headstage battery has no charge:
Attempting to charge the headstage during a recording will introduce 50Hz noise from the mains. If, at the start of the day, you find the headstage has been left on overnight, charge it during set-up – 5 to 20 minutes is sufficient to run the first mouse. The headstage can be also be charged between recordings. In addition, there is a battery with wires attached that can be used to charge the headstage during ABR recordings.

Broken electrode:

If one of the electrodes breaks, replace it with one of the same colour hanging on the wall. It is not necessary to fully remove the broken electrode if the electrode needs to be changed quickly – it is just necessary to unplug the damaged electrode from the headstage and, ideally, remove it from the electrode holder. The new electrode can then be inserted into the appropriate port in the headstage and placed in the holder. Once the electrodes have been placed into the scalp, check that the signal on the oscilloscope is clean and that there is a good ABR response. Fully remove broken electrode at the end of the session.

Noisy signal visible on the oscilloscope:
If there is a lot of noise visible on the trace on the oscilloscope, make sure the mouse is sufficiently anaesthetised. It should be possible to see the mouse's heartbeat. Replace the electrodes (ensuring the position of the electrodes is still correct). Check all the electrode connections; it may be worth repositioning the electrode holder or moving the electrode cables around a bit to see if this gets rid of some of the noise. Often, if the trace is only a little noisy, the recording settles down during recording.

No recordable ABR:

- If a heartbeat is present but there is no ABR, check the placement of the electrodes in the scalp. Make sure the speaker is producing sound (it's possible to hear clicks above 30 dB SPL if you open the recording chamber and listen while running Averager).
- The mouse may have a hearing impairment, in which case we don't expect an ABR.

If there is no heartbeat present and no ABR, check that the mouse is still alive. If the mouse has died, tell the x-ray team and contact the sick mouse team. If the mouse is alive, reposition the scalp electrodes.

Power cut:
Restart the computer and hardware when the power is back on. If there is time, re-run the experiment. If you can remember at which point the power cut occurred, you can start recording from that point. If you do this, you MUST give the ABR file a different name to the original file (giving the file the suffix '_1' would be appropriate under these conditions). Contact the person responsible for ABR before trying to upload the file(s) – they should be edited into one file before uploading.