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Data acquisition and analysis in brainstem evoked response audiometry in mice

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Bonn, 01. February 2018

GESCHZ -

Dear Dr. Bajaj,

Please find attached our revised manuscript entitled

"Data acquisition and analysis in
brainstem evoked response audiometry in mice"

by

Andreas Lundt, Julien Soos, Christina Henseler, Muhammad Imran Arshaad, Ralf Müller, Dan Ehninger, Jürgen Hescheler, Agapios Sachinidis, Karl Broich, Carola Wormuth, Anna Papazoglou, Marco Weiergräber

which we would like to resubmit for publication in the *Journal of Visualized Experiments (JoVE)*. We have addressed the remaining issues raised by the editor.

We hope our revised manuscript is suitable for publication in the *Journal of Visualized Experiments (JoVE)*.

Yours sincerely,

PD Dr. Dr. Marco Weiergräber

TITLE:

Data Acquisition and Analysis in Brainstem Evoked Response Audiometry in Mice

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KEYWORDS:

amplitude, auditory system, averaging, binaural, brainstem, click, hearing threshold, latency, monaural, systemic neurophysiology, tone burst, wavelet

SUMMARY:

Brainstem evoked response audiometry is an important tool in clinical neurophysiology. Nowadays, brainstem evoked response audiometry is also applied in the basic science and

preclinical studies involving both pharmacological and genetic animal models. Here we provide a detailed description of how auditory brainstem responses can be successfully recorded and analyzed in mice.

ABSTRACT:

Brainstem evoked response audiometry (BERA) is of central relevance in the clinical neurophysiology. As other evoked potential (EP) techniques, such as visually evoked potentials (VEPs) or somatosensory evoked potentials (SEPs), the auditory evoked potentials (AEPs) are triggered by the repetitive presentation of identical stimuli, the electroencephalographic (EEG) response of which is subsequently averaged resulting in distinct positive (p) and negative (n) deflections. In humans, both the amplitude and the latency of individual peaks can be used to characterize alterations in synchronization and conduction velocity in the underlying neuronal circuitries. Importantly, AEPs are also applied in basic and preclinical science to identify and characterize the auditory function in pharmacological and genetic animal models. Even more, animal models in combination with pharmacological testing are utilized to investigate for potential benefits in the treatment of sensorineural hearing loss (e.g., age- or noise-induced hearing deficits). Here we provide a detailed and integrative description of how to record auditory brainstem-evoked responses (ABRs) in mice using click and tone-burst application. A specific focus of this protocol is on pre-experimental animal housing, anesthesia, ABR recording, ABR filtering processes, automated wavelet-based amplitude growth function analysis, and latency detection.

INTRODUCTION:

A central aspect of brain physiology is its capability to process environmental information resulting in different intrinsic or extrinsic output, such as learning, memory, emotional reactions, or motoric responses. Various experimental and diagnostic approaches can be used to characterize the electrophysiological responsiveness of individual neuronal cell types or clusters/ensembles of neurons within a stimulus-related neuronal circuitry. These electrophysiological techniques cover different spatiotemporal dimensions on the micro-, meso- and macroscale[1]. The microscale level includes voltage and current clamp approaches in different patch-clamp modes using, for instance, cultured or acutely dissociated neurons[1]. These in vitro techniques allow for the characterization of individual current entities and their pharmacological modulation[2,3]. An essential drawback, however, is the lack of systemic information as regards micro- and macrocircuitry information integration and processing. This impairment is partially overcome by in vitro techniques of the mesoscale, such as multielectrode arrays which allow for simultaneous extracellular multielectrode recordings not only in cultured neurons but also in acute brain slices[4,5]. Whereas microcircuitries can be preserved in the brain slices to a specific extent (e.g., in the hippocampus), long-range interconnections are typically lost[6]. Ultimately, to study the functional interconnections within neuronal circuitries, systemic in vivo electrophysiological techniques on the macroscale are the method of choice[7]. These approaches include, among other things, surface (epidural) and deep (intracerebral) EEG recordings which are carried out in both humans and animal models[1]. EEG signals are predominantly based on the synchronized synaptic input on pyramidal neurons in different cortical layers that can be inhibitory or excitatory in principal, despite the general predominance of excitatory input[8]. Upon synchronization, excitatory postsynaptic potential-based shifts in extracellular electrical fields are

summed to form a signal of sufficient strength to be recorded on the scalp using surface electrodes. Notably, a detectable scalp recording from an individual electrode requires the activity of ten thousand of pyramidal neurons and a complex armamentarium of technical devices and processing tools, including an amplifier, filtering processes (low-pass filter, high-pass filter, notch filter), and electrodes with specific conductor properties.

In most experimental animal species (i.e., mice and rats), the human-based scalp EEG approach is technically not applicable, as the signal generated by the underlying cortex is too weak due to the limited number of synchronized pyramidal neurons[9–11]. In rodents, surface (scalp) electrodes or subdermal electrodes are thus severely contaminated by electrocardiogram and predominately electromyogram artifacts that make high-quality EEG recordings impossible[9,11,12]. When using unanesthetized freely moving mice and rats, it is therefore mandatory to directly record either from the cortex via epidural electrodes or from the deep, intracerebral structures to ensure the direct physical connection of the sensing tip of the lead/implanted electrode to the signal-generating neuronal cell clusters. These EEG approaches can be carried out either in a restraining tethered system setup or using the nonrestraining implantable EEG radio telemetry approach[9–11]. Both techniques have their pros and cons and can be a valuable approach in the qualitative and quantitative characterization of seizure susceptibility/seizure activity, circadian rhythmicity, sleep architecture, oscillatory activity, and synchronization, including time-frequency analysis, source analysis, etc.[9,10,13–17].

Whereas tethered systems and radio telemetry allow for EEG recordings under restraining/semirestraining or nonrestraining conditions, respectively, related experimental conditions do not match the requirements for ABR recordings. The latter demand for defined acoustic stimuli which are presented repetitively over time with defined positions of a loudspeaker and experimental animal and controlled sound pressure levels (SPLs). This can be achieved either by head fixation under restraining conditions or following anesthesia[18,19]. To reduce the experimental stress, animals are normally anesthetized during ABR experimentation, but it should be considered that anesthesia can interfere with ABRs[19,20].

As a general characteristic, the EEG is built up of different frequencies in a voltage range of 50–100 µV. Background frequencies and amplitudes strongly depend on the physiological state of the experimental animal. In the awake state, beta (β) and gamma (γ) frequencies with lower amplitude predominate. When animals become drowsy or fall asleep, alpha (α), theta (θ), and delta (δ) frequencies arise, exhibiting increased EEG amplitude[21]. Once a sensory channel (e.g., the acoustic pathway) is stimulated, information propagation is mediated via neuronal activity through the peripheral and central nervous system. Such sensory (e.g., acoustic) stimulation triggers so-called EPs or evoked responses. Notably, event-related potentials (ERPs) are much lower in amplitude than the EEG (i.e., a few microvolts only. Thus, any individual ERP based on a single stimulus would be lost against the higher-amplitude EEG background. Therefore, a recording of an ERP requires the repetitive application of identical stimuli (e.g., clicks in ABR recordings) and subsequent averaging to eliminate any EEG background activity and artifacts. If ABR recordings are done in anesthetized animals, it is easy to use subdermal electrodes here.

Principally, AEPs include short-latency EPs, which are normally related to ABRs or BERA, and further, later-onset potentials such as midlatency EPs (midlatency responses [MLR]) and long-latency EPs[22]. Importantly, disturbance in the information processing of the auditory information is often a central feature of neuropsychiatric diseases (demyelinating diseases, schizophrenia, etc.) and associated with AEP alterations[23–25]. Whereas behavioral investigations are only capable of revealing functional impairment, AEP studies allow for precise spatiotemporal analysis of auditory dysfunction related to specific neuroanatomical structures[26].

ABRs as early, short-latency acoustically EPs are normally detected upon moderate to high-intense click application, and there may occur up to seven ABR peaks (W_I–W_{VII}). The most important waves (W_I–W_V) are related to the following neuroanatomical structures: W_I to the auditory nerve (distal portion, within the inner ear); W_{II} to the cochlear nucleus (proximal portion of the auditory nerve, brainstem termination); W_{III} to the superior olivary complex (SOC); W_{IV} to the lateral lemniscus (LL); W_V to the termination of the lateral lemniscus (LL) within the inferior colliculus (IC) on the contralateral side[27] (**Supplementary Figure 1**). It should be noted that W_{II}–W_V are likely to have more than one anatomical structure of the ascending auditory pathway contributing to them. Notably, the exact correlation of peaks and underlying structures of the auditory tract is still not fully clarified.

In audiology, ABRs can be used as a screening and diagnostic tool and for surgical monitoring[28,29]. It is most important for the identification of dysacusis, hypacusis, and anacusis (e.g., in age-related hearing loss, noise-induced hearing loss, metabolic and congenital hearing loss, and asymmetric hearing loss and hearing deficits due to deformities or malformations, injuries, and neoplasms)[28]. ABRs are also relevant as a screening test for hyperactive, intellectually impaired children or for other children who would not be able to respond to conventional audiometry (e.g., in neurological/psychiatric diseases such as ADHD, MS, autism etc.[29,30] and in the development and surgical fitting of cochlear implants[28]. Finally, ABRs can provide valuable insight into the potential ototoxic side-effects of neuropsychopharmaceuticals, such as antiepileptics[31,32].

The value of the translation of neurophysiological knowledge obtained from pharmacological or transgenic mouse models to humans has been demonstrated in numerous settings, particularly on the level of ERPs in auditory paradigms in mice and rats[33–35]. New insight into altered early AEPs and associated changes in auditory information processing in mice and rats can thus be translated to humans and is of central importance in the characterization and endophenotyping of auditory, neurological, and neuropsychiatric diseases in the future. Here we provide a detailed description of how ABRs can be successfully recorded and analyzed in mice for basic scientific, toxicological, and pharmacological purposes.

PROTOCOL:

All animal procedures were performed according to the guidelines of the German Council on Animal Care and all protocols were approved by the local institutional and national committee on animal care (Landesamt für Natur, Umwelt, und Verbraucherschutz, State Office of North Rhine-Westphalia, Department of Nature, Environment and Consumerism [LANUV NRW], Germany).

The authors further certify that all animal experimentation was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 or the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, or the European Communities Council Directive of November 24, 1986 (86/609/EEC) and of September 22, 2010 (2010/63/EU). Specific effort was made to minimize the number of animals used and their suffering (3R [replacement, reduction, and refinement] strategy).

1) Experimental animals

1.1) Selection of experimental animals and species

1.1.1) Perform ABR studies in rodents/rodent models (i.e. mice or rats) that fulfill the requirements of homology, isomorphism, and predictability related to a specific human disease. This is of specific importance in terms of basic aspects in translational neuroscience.

NOTE: Consider that available miscellaneous mouse and rat strains can show differences in basic physiological and pathophysiological characteristics[36–38]. These mouse/rat line-related specificities have to be taken into account in experimental planning.

1.1.2) Consider mouse- and rat-strain-specific alterations in physiology and pharmacology that might have an impact on electrophysiological experiments (e.g. altered anesthetic sensitivities, circadian rhythmicity, [audiogenic] seizure susceptibility, age, and genetic background)[39–42].

1.1.3) Include the gender-specific stratification in the study design. Remember that the estrous cycle can severely affect anesthetic susceptibility, central rhythmicity, circadian dependency, and seizure activity (auditory seizures) and sensory (auditory) information processing[43–45]. Thus, perform a gender-specific analysis.

NOTE: Restrict to male mice if the financial and experimental capacity is limited, although various neurophysiological parameters from normally cycling females do not seem to exhibit increased variability compared to males[46].

1.2) Animal housing and handling

1.2.1) House mice or rats in individually ventilated cages inside an animal facility.

1.2.2) Move the experimental animals from the animal facility to ventilated cabinets located in special lab rooms intended for anesthesia, ABR electrode placement, and ABR recordings.

1.2.3) Make sure that the animals are housed in a ventilated cabinet under standard environmental conditions (i.e., with a temperature of 21 ± 2 °C, 50%–60% relative humidity, and a conventional 12/12 h light/dark cycle). Allow the animals to acclimatize and adapt to this circadian pattern for at least 14 days prior to subsequent experimentation.

1.2.4) Use clear polycarbonate cages type II (26.7 cm x 20.7 cm x 14.0 cm, an area of 410 cm^2) for housing mice in groups of 3–4 and use clear polycarbonate cages type III (42.5 cm x 26.6 cm x 18.5 cm, an area of 800 cm^2) for rats. Provide *ad libitum* access to drinking water and standard food pellets.

1.2.5) Avoid separation/isolation of the experimental animals prior to and after ABR recordings as isolation can exert severe stress affecting experimental results. Thus, place the animals back into their home cage following anesthesia, ABR electrode placement, and ABR recordings.

1.2.6) Do not apply open housing conditions as they are subject to a variety of experimental drawbacks, particularly in auditory studies. Ventilated cabinets, instead, protect from acoustic stress prior to and in between experimental auditory procedures which could otherwise lead to sensorineural hearing loss (e.g., noise-induced hearing loss) and thus affect results.

1.2.7) Utilize mouse- and rat-specific sanitary, anesthetic, and technical equipment so that neither mice nor rats can sense the presence of each other as mutual sensory perception of rivaling species may give rise to avoidable confounding factors in the studies.

2) Mouse anesthesia

2.1) Perform anesthesia using injectable anesthetics. Prepare a combination of ketamine hydrochloride (rodent dosage: 100 mg/kg) and xylazine hydrochloride (rodent dosage: 10 mg/kg) in 0.9% NaCl or Ringer solution and inject the animal intraperitoneally based on its body weight.

NOTE: Inhalation narcosis via isoflurane is not recommended as the ABR procedure normally requires a sound attenuating cubicle and Faraday cage, resulting in spatial limitations within the recording setup. Although many anesthetics act on the NMDA system and obviously influence ABR recording outcomes, a non-anesthetic restraining approach in ABR recordings is not recommended as restraining procedures under consciousness induce dramatic stress to the animal, with severe subsequent artifact formation in ABRs.

2.2) Observe the animals carefully for the depth of anesthesia by performing a tail pinch, foot pinch, and monitoring respiration rate (mice: 150–220 breaths/min). Check for possible gasping and counteract if necessary.

NOTE: Different mouse lines or pharmacological mouse models can exhibit different sensitivities to anesthesia. The same holds true for mutant mouse models. Endotracheal intubation is not a must in this experimental setting and not recommended. As intubation increases the risk of trauma to the trachea and infection, the benefit/risk of endotracheal intubation during the ABR procedure is negative.

3) General aspects of perianesthetic arrangements and instrumentation

3.1) Apply supplemental warmth during and after ABR recordings using a homeothermic heating

blanket to maintain the animal's body core temperature. Maintain the latter at 36.5–38.0 °C (98.6–100.4 °F).

NOTE: Hypothermia is a risk factor in small rodents due to their high ratio of body surface (mouse body surface = 10.5 x (weight in g)$^{2/3}$; rat body surface = 10.5 x (weight in g)$^{2/3}$) to the body volume.

3.2) Cover the animal's eyes with petroleum-based artificial tear ointment or 5% dexpanthenol during the entire ABR recording process to avoid corneal desiccation. Continue this procedure until the blinking reflex is fully restored.

3.3) Sterilize the experimental instruments (see the **Table of Materials**) using an autoclave or disinfectants.

NOTE: The usage of a heat-based surgical instrument sterilizer with glass beads is recommended.

3.4) For exact ABR electrode placement, use a binocular surgical magnification microscope with a cold light source for the intense illumination via flexible or self-supporting movable light guides.

3.5) Use a clean laboratory coat, a facemask, a head cover, and sterile gloves during experimental animal handling and experimentation.

NOTE: Optimal instruments and supplies can vary between labs and must meet lab-specific and institutional standards.

4) ABR recordings

NOTE: The protocol described here is based on a commercially available ABR system for monaural and binaural recordings. Importantly, the scientific question to be addressed must meet the technical specifications of the ABR system used. ABR analysis of binaural recordings, for example, can be used to investigate the lateral coding of auditory stimuli in the auditory pathway and to study peripheral lateral asymmetry in neuropsychiatric diseases.

4.1) Perform a calibration of stimulation frequencies on each day of recording by placing a microphone connected to a preamplifier and the processing system (see the **Table of Materials**) inside the sound attenuating cubicle at the location with the correct orientation where the experimental murine ear will be positioned.

4.1.1) Turn on the preamplifier connected to the microphone at least 5 min prior to the calibration to allow for the equilibration of the system.

4.1.2) Turn on the oscilloscope.

4.1.3) Position the microphone connected to a preamplifier inside the sound attenuating cubicle to mimic the experimental murine ear.

4.1.4) Open the commercially available processing and acquisition software (see the **Table of Materials**).

4.1.5) Select the calibration **Cal200K** file within the software to activate the calibration-configuration mode and choose parameters according to the experimental conditions.

4.1.6) Use the processor system to execute the calibration procedure. Make sure that the technical specifications of the microphone and loudspeaker in terms of SPL limits, frequency range, and distribution harmonize.

4.1.7) Select and start the predefined click stimulation protocol.

4.1.8) Run a single click SPL (preferably, the maximum SPL) to verify that the spectrum of sound stimuli as analyzed by online Fast Fourier Transformation (FFT) of the oscilloscope matches requirements (substantial energy in the 0.8–43 kHz range).

4.1.9) Select and start the predefined tone-burst stimulation protocol within the range of 1–42 kHz.

4.1.10) Confirm the frequency spectrum of the recorded acoustic test stimuli by using an oscilloscope and online FFT.

NOTE: Daily calibration of the system and stimulation frequencies is necessary to guarantee that the stimulation frequencies and SPLs are within acceptable working ranges.

4.2) Place the anesthetized mouse inside a sound attenuating cubicle lined with acoustical foam.

NOTE: The entire cubicle should be covered by a Faraday cage (custom-made meshed metal or a commercial one) to shield the ABR recordings from external electrical interference and protect them from noise.

4.3) For the recording of monaural brainstem-evoked auditory potentials, insert subdermal stainless-steel electrodes at the vertex, axial of the pinnae (positive [+] electrode) and ventrolateral of the right or left pinna (negative [-] electrode) depending on the ear to be measured. For binaural recordings, place the negative electrodes at both the right and left pinnae. Position the ground electrode at the hip of the animal (**Supplementary Figure 1**).

4.3.1) Prior to the insertion, form a hook shape at the tip of the stainless-steel electrode so that subdermal fixation of the electrodes is guaranteed[47].

4.4) Perform impedance measurements of all electrodes prior to each recording to verify proper electrode positioning/conductivity. Use the impedance check button on the four-channel headstage to verify each electrode impedance level.

NOTE: Impedance should be less than 5 kΩ.

4.5) Record ABRs under free-field conditions using a single loudspeaker (frequency bandwidth, for instance, at 1–65 kHz) placed 10 cm opposite to the rostrum of the animals (the loudspeaker's leading edge perpendicular to the mouse's interaural axis). Make sure that the position of the mouse head/mouse ears is that of the calibration microphone, depending on the chosen specific distance between the loudspeaker and the microphone during calibration.

NOTE: Instead of free-field conditions, ear tubing can also be used. However, special precautions and tests are necessary to determine SPLs in these settings.

4.6) Program the stimulus protocols for the clicks and tone bursts using self-programmed or commercially available software (see the **Table of Materials**). The individual stimulus parameters listed below need to be added to the related graphical user interface.

4.6.1) Start with the configuration of the click stimulus entity (i.e., a 100 µs duration stimulus with alternating polarity [switching between condensation and rarefaction] and the substantial energy in the 0.8–43 kHz range). Use this stimulus entity to analyze and determine click thresholds, ABR symmetry of the left and right ear, ABR W (I - IV) amplitudes, and W (I - IV) latencies later on.

4.6.2) Initiate the software and use the configuration window to add the click stimulus parameters. Click **Execute** to run the protocol.

4.6.3) Continue with the configuration of the second stimulus entity, which is a 4.5 ms tone burst (transient sinusoidal pulse) of alternating polarity with Hann envelope rise and fall times of 1.5 ms each (gate/ramp time duration). Consider a minimum tone burst duration of 3 ms, particularly for low-frequency tone bursts. Use this stimulus to analyze and identify frequency-specific hearing thresholds in all genotypes.

4.6.4) Similar to step 4.6.2, use the configuration window to add tone burst stimulus parameters and click **Execute** to run the protocol (as stated by the manufacturer[48]).

4.6.5) For tone burst studies, program the appropriate frequency range to be tested depending on the scientific question (e.g., from 1–42 kHz in 6 kHz steps). Make sure that the frequency ranges to be applied meet the technical capabilities of the loudspeaker (in this case, a multifield magnetic speaker with a frequency bandwidth of 1–65 kHz for free- or closed-field conditions).

4.6.6) For averaging, set the number of sequential acoustic stimuli (clicks or tone bursts), for instance, at 300x with a rate of 20 Hz.

4.6.7) Increase the SPLs in 5 dB steps for clicks and 10 dB steps for tone bursts, starting from 0 dB up to 90 dB (increasing SPL mode).

NOTE: Both increasing and decreasing SPL modes have been described in the literature. SPL step size might be adapted due to scientific questions.

4.7) Determine an ABR data acquisition duration of 25 ms, starting with a 5 ms baseline period prior to the individual acoustic stimulus onset (pre-ABR baseline) and exceeding a 10 ms ABR section by another 10 ms baseline (post-ABR baseline) (**Supplementary Figure 1**).

4.8) Apply an appropriate sampling rate for ABR data acquisition (e.g., 24.4 kHz) and bandpass filter (high pass: 300 Hz, low pass: 5 kHz) using a 6-pole Butterworth filter. Activate the notch filter if necessary.

NOTE: Sampling rate and filter characteristics might be adapted due to experimental requirements.

4.9) Transfer the resultant bioelectrical signals recorded from the subdermal electrodes to a head stage and further forward to a preamplifier with appropriate amplification (e.g., 20-fold).

4.10) Use a specific ABR system processing software to coordinate loudspeaker control and ABR acquisition, processing, averaging, and data management.

4.11) Try to execute the entire ABR protocols (for click- and tone burst-evoked hearing thresholds, peak amplitude, and peak latency analysis, etc.) within about 45 min. This corresponds to the time of deep narcosis using 100/10 mg ketamine/xylazine intraperitoneally.

4.12) Make sure that the calibration, programming/adjustments for stimulus presentation and acquisition, filter settings, etc. are working as expected prior to anesthetizing the animal and performing the actual recording.

5) ABR analysis

5.1) Click- and tone burst-evoked ABR hearing threshold analysis

5.1.1) Perform automated threshold detection based on earlier publications to avoid potential inconsistencies in the ABR threshold determination by visual inspection/estimation[49–52].

5.1.2) Define three distinct time windows (TWs) to calculate the signal-to-noise ratio (SNR): TW_1 (0–5 ms), TW_2 (5–15 ms), and TW_3 (15–25 ms) (**Supplementary Figure 1**).

5.1.3) Calculate the noise standard deviation of the baseline within the two distinct TWs (i.e., TW_1 and TW_3) where no AEPs are observed. This calculation can be done using self-programmed software.

5.1.4) Calculate for each SPL measurement within an ABR record setting both the mean and the standard deviation for the pooled data of TW_1 and TW_3.

5.1.5) Reset all recording samples individually by the corresponding calculated mean to remove any DC offset.

5.1.6) For hearing threshold determination, identify the lowest SPL (dB) where at least one wave amplitude value (W_I–W_{IV}) in the ABR response time window (TW_2) exceeded the fourfold of the previously calculated standard deviation.

NOTE: If no ABR wave (W_I–W_{IV}) was detected for frequency threshold analysis at the maximum SPL, a nominal threshold level of 100 dB is assigned to the ear.

5.2) ABR wave amplitude and wave latency analysis

5.2.1) Conduct a wavelet-based approach using the Mexican hat wavelet to determine the temporal-sequential arrangement of positive (p) waves (peaks) as well as the negative (n) waves (pits) using a default wavelet by the continuous wavelet transform (CWT)-based pattern-matching algorithm[52] (**Supplementary Figure 1**).

5.2.1.1) Mathematically, the CWT is represented as follows[53].

$$C(a,b) = \int_R s(t)\psi_{a,b}(t)dt, \psi_{a,b}(t) = \frac{1}{\sqrt{a}}\psi\left(\frac{t-b}{a}\right), a \in R^+ - \{0\}, b \in R$$

Here, *s(t)* is the signal, *a* is the scale, *b* is the translation, $\psi(t)$ is the mother wavelet, $\psi_{a,b}(t)$ is the scaled and translated wavelet, and *C* is the 2D matrix of wavelet coefficients.

5.2.2) Initially, use the 55-dB measurement of each ABR run to identify the best scale parameters for each wave to be passed to the CWT, which results in three classes: scales 0.5–4 for all n-waves, 0.5–6 for all p-waves, and 0.5–12 for W_{IV} as this is the broadest wave within the samples.

NOTE: The 55 dB SPL was chosen as waves are most prominent here and can be reliably detected.

5.2.3) Prove all classes to reliably detect the correct temporal collocation of W_I–W_{IV} within all 55 dB measurements.

5.2.4) To determine ABR W_I–W_{IV} in the accurate temporal order within the 55 dB measurement, p-peaks and n-peaks (pits) are identified in a fixed sequence using relative positions of previously identified peaks to limit the time window of subsequent scans.

5.2.5) Once all nine peaks are identified at 55 dB, use related values as starting points for the temporal search frame for the adjacent sound pressure measurements (50 dB and 60 dB) before the identification of peaks 1–9 is repeated.

5.2.6) In this manner, determine p- and n-peaks of all dB levels (55–0 dB and 60–90 dB) if possible.

Once a p- and n-peak is no longer identified by the wavelet analysis, its temporal arrangement is set by calculating the temporal offset of the peak to any other peak identified in the previous dB level.

5.2.7) Applying the temporal offset to peaks to any other p- and n-peak within the current decibel level results in a maximum of eight determined temporal positions for the undefined peaks whereof the mean is taken as the closest approximation.

5.2.8) To evaluate the amplitude growth function and latency comparison of all waves (W_I–W_{IV}), characterize the maximum amplitudes and mean latencies of each of the p-peaks within the time frame of the related n-peaks.

5.2.9) Visually check all results based on the self-programmed automatic wavelet tool afterward, and, if necessary, exclude individual ABR runs from the statistics if they do not meet the strict inclusion/quality criteria.

NOTE: In both automated analysis and visual inspection of ABRs, a double-blinded approach is recommended.

6) Post-operative care and post-ABR treatment

6.1) Continuously monitor the animals until they have regained consciousness and are able to maintain sternal recumbency.

6.2) Do not return an animal that has undergone ABR recordings to the company of other animals until it has fully recovered.

6.3) Inject carprofen (mouse: 1x 5–10 mg/kg, subcutaneously; rat: 1x 2.5–5.0 mg/kg, subcutaneously) for post-operative pain treatment.

NOTE: Long-lasting pain treatment is not required as ABR recording electrodes are inserted subcutaneously.

6.4) Postoperatively, feed moistened pellets in order to facilitate food uptake. Carefully observe food (~15 g/100 g of body weight/day; ~5 g/24 h) and water (~15 mL/100 g of body weight/day; ~5 mL/24 h) consumption.

6.5) Monitor the animals closely for the return of their normal postures and behavior.

NOTE: Systemic administration of antibiotics such as enrofloxacin or trimethoprim-sulfonamide is not recommended here, as subdermal electrode placement is of only minimal invasiveness. Application of antibiotics should be restricted unless signs of local or generalized inflammation occur.

6.6) Follow-up postexperimental recovery after ABR recordings by controlling the animal's body weight.

REPRESENTATIVE RESULTS:

Click- and tone burst-evoked ABR recordings can be used to evaluate hearing threshold differences, amplitude growth function, and latency comparison. Click-evoked ABRs in the SPL increasing mode are depicted in **Figure 1** for controls and two exemplary mutant mouse lines which are deficient for the $Ca_v3.2$ T-type voltage-gated Ca^{2+} channel (i.e., $Ca_v3.2^{+/-}$ and $Ca_v3.2$ null mutants [$Ca_v3.2^{-/-}$]). As outlined above, a gender-specific investigation is generally recommended, due to sex-specific differences in auditory parameters in humans[54,55] and mice[56,57]. ABRs to free-field click (0.1 ms) and tone burst (1–42 kHz in 6 kHz steps, 4.5 ms in total with a 1.5 ms ramp time) acoustic stimuli were recorded as described in the protocol. Note that the vertex-positive potentials are plotted as upward deflections as depicted in representative click-evoked recordings for female $Ca_v3.2^{+/+}$ (**Figure 1A**), $Ca_v3.2^{+/-}$ (**Figure 1B**), and $Ca_v3.2^{-/-}$ mice (**Figure 1C**). In this setting, representative ABRs in females suggested an increased click-evoked ABR hearing threshold and altered amplitude growth function in female $Ca_v3.2^{-/-}$ mice compared to $Ca_v3.2^{+/+}$ and $Ca_v3.2^{+/-}$ animals. The same tendency was observed for males which suggested an increased click-evoked ABR thresholds and reduced amplitudes in $Ca_v2.3^{-/-}$ compared to controls and heterozygous $Ca_v3.2^{+/-}$ mice. Exemplary tone burst-evoked ABRs are depicted in **Figure 2** for female $Ca_v3.2^{+/+}$, $Ca_v3.2^{+/-}$, and $Ca_v3.3^{-/-}$ mice (all animals were 20 weeks of age).

As a first step in analyzing general hearing performance, click-evoked ABRs for different SPLs (0–90 dB) were investigated using the automated ABR threshold detection system described in section 5 of the protocol (**Figure 3**). The analyzed animals were age matched as aging can have a dramatic impact on sensorineural hearing loss[58,59]. Next, potential alterations in ABR threshold levels evoked by different tone burst frequencies (1–42 kHz, **Figure 4**) were analyzed. In the exemplary mouse lines, $Ca_v2.3^{+/-}$ and $Ca_v3.2^{-/-}$ exhibited increased click- and tone burst-related hearing thresholds compared to controls (all animals were 20 weeks of age).

Using the wavelet-based approach outlined above, click-evoked ABR amplitude growth function and ABR waveform latency analysis were carried out (**Figure 5** and **Figure 6**, respectively). The latter allows insight into the possible spatiotemporal influence of the gene of interest on auditory information processing within the inner ear and brainstem.

FIGURE LEGENDS:

Figure 1: Click-evoked ABRs in controls and mutant mice ($Ca_v3.2^{+/-}$, $Ca_v3.2^{-/-}$). Representative ABRs obtained from (**A**) $Ca_v3.2^{+/+}$, (**B**) $Ca_v3.2^{+/-}$, and (**C**) $Ca_v3.2^{-/-}$ female mice upon click stimulation in the increasing SPL mode (from 0–90 dB with 5 dB SPL steps). For averaging, each stimulus entity was applied 300 times at 20 Hz. The acoustic stimulus onset is indicated by a vertical red line. This figure is modified from Lundt et al.[60].

Figure 2: Tone burst-evoked ABRs in controls and mutant mice ($Ca_v3.2^{+/-}$, $Ca_v3.2^{-/-}$). Representative ABRs from (**A**) $Ca_v3.2^{+/+}$, (**B**) $Ca_v3.2^{+/-}$, and (**C**) $Ca_v3.2^{-/-}$ female mice following tone

bursts of 1–42 kHz (6 kHz steps) at an SPL of 80 dB. For averaging, each stimulus entity was presented 300 times at 20 Hz. The acoustic stimulus onset is indicated by a vertical red line. This figure is modified from Lundt et al.[60].

Figure 3: Click-evoked ABR-based hearing thresholds in controls and mutant mice ($Ca_v3.2^{+/-}$, $Ca_v3.2^{-/-}$). Click-evoked audiometric hearing threshold of (**A**) female and (**B**) male $Ca_v3.2^{+/+}$ (female: *n* = 12; male: *n* = 13), $Ca_v3.2^{+/-}$ (female: *n* = 10; male: *n* = 9), and $Ca_v3.2^{-/-}$ mice (female: *n* = 10; male: *n* = 9). Data are plotted as mean ± SEM. Statistical significances were determined using α-level = 0.05 and p-values defined as **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001. This figure is modified from Lundt et al.[60].

Figure 4: Tone burst-evoked ABR-based hearing thresholds in controls and mutant mice ($Ca_v3.2^{+/-}$, $Ca_v3.2^{-/-}$). 1–42 kHz (6 kHz steps) tone burst-evoked ABR-based audiometric hearing thresholds for $Ca_v3.2^{+/+}$ (female: *n* = 12; male: *n* = 12; ▲), $Ca_v3.2^{+/-}$ (female: *n* = 10; male: *n* = 8; ■), and $Ca_v3.2^{-/-}$ animals (female: *n* = 10; male: *n* = 9; ○). Data are plotted as mean ± SEM. Statistical significances were determined using α-level = 0.05 and p-values defined as **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001. This figure is modified from Lundt et al.[60].

Figure 5: Amplitude growth function on click-based ABR recordings in controls and mutant mice ($Ca_v3.2^{+/-}$, $Ca_v3.2^{-/-}$). W_I–W_{IV} amplitude (in microvolts) plotted against an increasing SPL (in decibels) for click-evoked ABR wave analysis in $Ca_v3.2^{+/+}$ (female: *n* = 12; male: *n* = 11; black line representing the approximated control curve including the 95% confidence interval in grey), $Ca_v3.2^{+/-}$ (female: *n* = 8; male: *n* = 7; ■), and $Ca_v3.2^{-/-}$ animals (female: *n* = 7; male: *n* = 9; ○). Both $Ca_v3.2^{-/-}$ female and male mice exhibit significantly delayed increase in the amplitude growth across the increasing SPLs for (**A** and **B**) W_I, (**C** and **D**) W_{II}, and (**G** and **H**) W_{IV} compared to $Ca_v3.2^{+/+}$ and $Ca_v3.2^{+/-}$ mice. (**E** and **F**) For W_{III}, only $Ca_v3.2^{-/-}$ male mice displayed a significant delay in amplitude growth across the increasing SPL compared to female $Ca_v3.2^{-/-}$ animals. Data are presented as mean ± SEM. Statistical significances were determined using α-level = 0.05 and p-values defined as **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001. This figure is modified from Lundt et al.[60].

Figure 6: Latency analysis upon click-evoked ABR recordings in controls and mutant mice ($Ca_v3.2^{+/-}$, $Ca_v3.2^{-/-}$). Latencies (in milliseconds) for each ABR wave (W_I–W_{IV}) at 65 dB SPL are depicted for $Ca_v3.2^{+/+}$ (female: *n* = 12; male: *n* = 11), $Ca_v3.2^{+/-}$ (female: *n* = 8; male: *n* = 7), and $Ca_v3.2^{-/-}$ mice (female: *n* = 8; male: *n* = 9). Data are depicted as mean ± SEM. Statistical significances were determined using α-level = 0.05 and p-values defined as **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001. This figure is modified from Lundt et al.[60].

Supplementary Figure 1: ABR architecture and electrode positioning. (**A**) Representative ABR recording at 65 dB SPL. The initial baseline (TW_1, 5 ms) was followed by the test stimulus (click or tone burst) and TW_2 (10 ms) containing the early brainstem-evoked potentials. TW_2 was followed by another baseline (TW_3, 10 ms). The baseline periods were used to calculate the SD of the baseline noise. Whenever an individual ABR wave (W_I–W_{IV}) amplitude exceeded the SD of the baseline noise in fourfold, the hearing threshold was reached. For wave amplitude and latency

comparison, a “Mexican hat”-based wavelet approach was carried out to automatically detect negative peaks (blue-yellow striped lines) and positive peaks (red-grey striped lines). Green crosses indicate the absolute maximum ABR wave amplitudes and do not display approximated values based on the wavelet approach. (**B**) For the ABR recordings, subdermal stainless-steel electrodes with a hook-shaped tip were used. The reference electrode was placed at the left hip, the positive (+) electrode was positioned at the vertex (axial of the pinnae), and the negative (-) electrode was inserted ventrolateral of the right pinna depending on whether a monaural or binaural recording was carried out. This figure is modified from Lundt et al.[60].

DISCUSSION:

This protocol provides a detailed and integrative description of how to record auditory evoked brainstem responses in mice. It puts specific focus on animal pretreatment, anesthesia, and potential methodological confounding factors. The latter include, among others, gender, mouse line, age, and housing conditions. It should be noted that all these factors can have an impact on sensorineural hearing loss and fundamental aspects of auditory information processing. Thus, appropriate stratification of auditory profiling studies is mandatory.

The instrumentation of AEP recordings has tremendously evolved in the last 50–60 years, and nowadays, commercial ABR recording systems are available which have enhanced and simplified the application of the technique but have also introduced new pitfalls. Some of these aspects are discussed here. First, the user should get used to the ABR system, that is, the instrumentation composed of desktop or laptop computer, the preamplifier, the amplifier, the electrode input box, and potential transducers (e.g., loudspeakers, insert earphones, supra-aural headphones, and bone oscillators). Notably, recording conditions are of central importance. Due to their high susceptibility, ABR recordings need to be shielded to protect them from contamination with external electrical noise and to guarantee an adequate signal-to-noise ratio.

Another important aspect is the instrumentation itself (e.g., the stimulus generator, transducers, and triggers). The most commonly used types of stimuli in mice are 100 µs clicks and short-duration tone bursts with a modulated amplitude and/or frequency properties. Transducers can present a variety of acoustic stimuli either to one ear or to both ears. Here we have presented ABR results using a single loudspeaker rostral to the experimental animal. However, other approaches are also possible, including tubal-style insert earphones either in one ear or both ears. Supra-aural headphones as used in humans are not feasible in mice. As literature illustrates, different approaches can be successful, and they should be adapted depending on the experimental needs. Special attention has to be paid to the accurateness of the trigger which is essential for signal averaging as this digital pulse determines when each individual stimulus is presented. For proper recordings, the trigger and stimulus onset must be synchronous, representing time point zero. Commercially available ABR recording systems normally include self-contained triggers when the individual stimuli are presented. In many systems, there are external inputs which allow a connection from an external stimulus generator and an associated trigger. In both cases, it turned out to be valuable to control the stimulus and trigger characteristics using an external oscilloscope. Special attention also must be paid to the acquisition parameters (e.g., differential amplification, filtering, analog versus digital filters, filter

designs, and parameters of signal averaging). Notably, parameters presented in the protocol presented here fit the experimental requirements of the exemplary results depicted above. However, adaptations, for instance in the sampling rate, number of stimuli applied for averaging, and their application frequency, might be necessary depending on the experimental settings.

Finally, some brief comments should be made on electrode impedance, electrode types, and electrode placement. The electrodes act like antennas, picking up voltage changes from below the skin. Subcutaneous electrode placement is mandatory as the mere application of electrodes on the skin or scalp is not suitable because of the resistance of the outer skin layer (i.e., the stratum corneum). Whereas in humans the electrical conductivity is normally improved by abrading dead skin cells and the application of an electrolyte gel or paste, this is usually not done and suitable in mice where subdermal electrodes are used. The interface of electrode and skin forms the electrode impedance which includes the electrical properties of the conductor in total. The conductor properties include the material properties of the electrode and the surface area of the contacting electrode, properties of the tissue including the debris (oil, dirt, sweat, etc.), and the electrolyte solution. The electrode material includes silver, gold, platinum, lead, tin, and stainless steel with low impedance and low electrode potentials. Care has to be taken that the electrode material is inert under recording conditions. With silver, this is achieved by using so-called complex electrodes (i.e., silver-silver chloride [Ag-AgCl] electrodes). In this case, the electrical double layer allows for a free exchange of ions which further reduces impedance. It is often recommended that electrode impedance should not exceed 5 kΩ and that the impedance of the individual electrodes (at least three) is comparable. It is also recommended that the interelectrode impedance should be below 2 kΩ. The recording electrode represents a long metal wire with an insulating coating. The wire electrode is connected via a plug to the recording equipment, in most cases the preamplifier/amplifier. In mice, the other end of the electrode wire is usually buildup of a needle electrode that might be left straight or—better—arcuated. Other electrode types, such as disk- or cup-shaped ones, no matter whether they are for reuse or pregelled disposable, are restricted to use in humans and not applicable to mice.

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The authors have nothing to disclose.

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Figure 1

Click here to access/download;Figure;Figure_1_MWe.pdf

Figure 1 (Lundt et al., 2018)

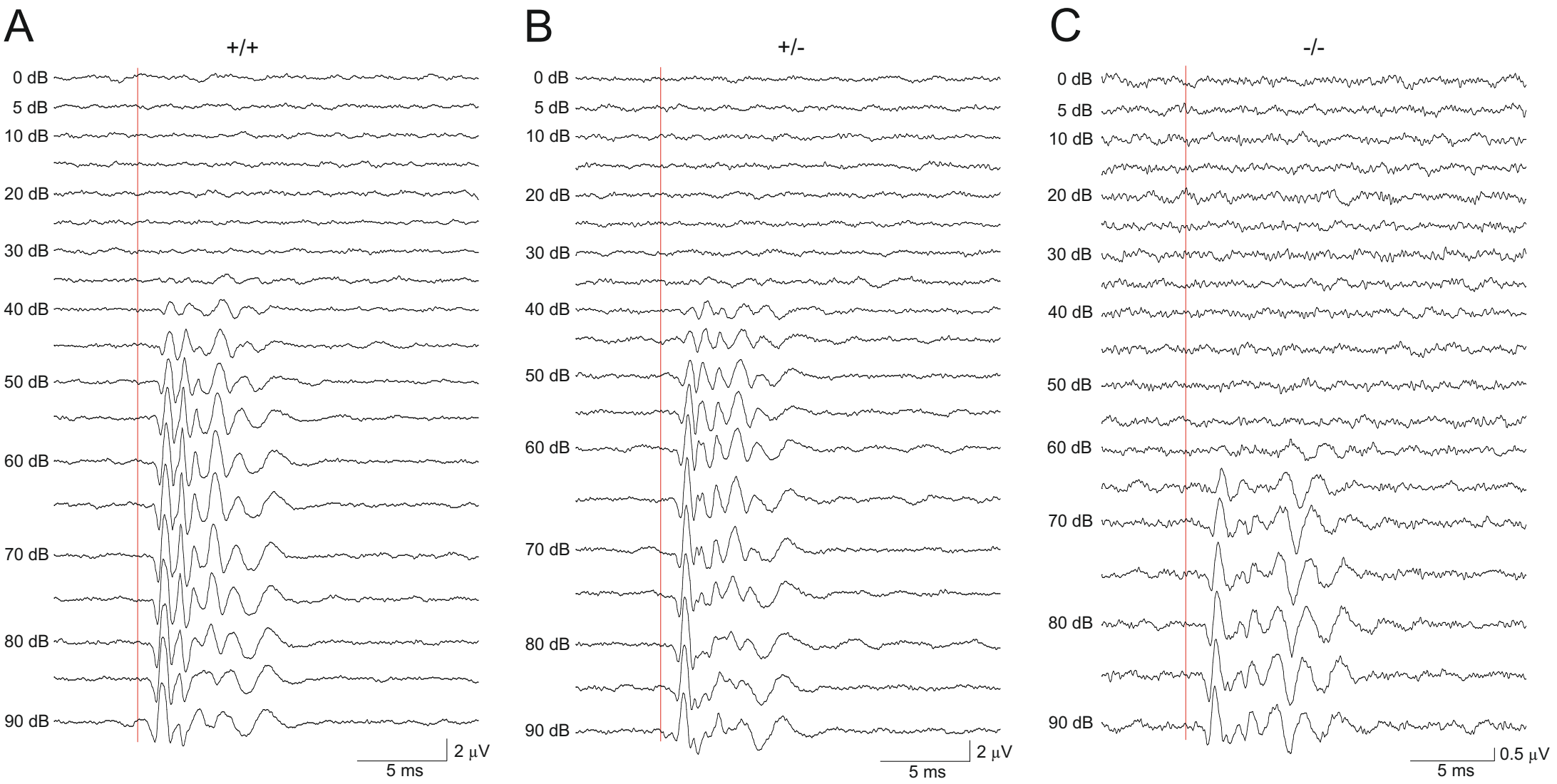

Figure 2 (Lundt et al., 2018)

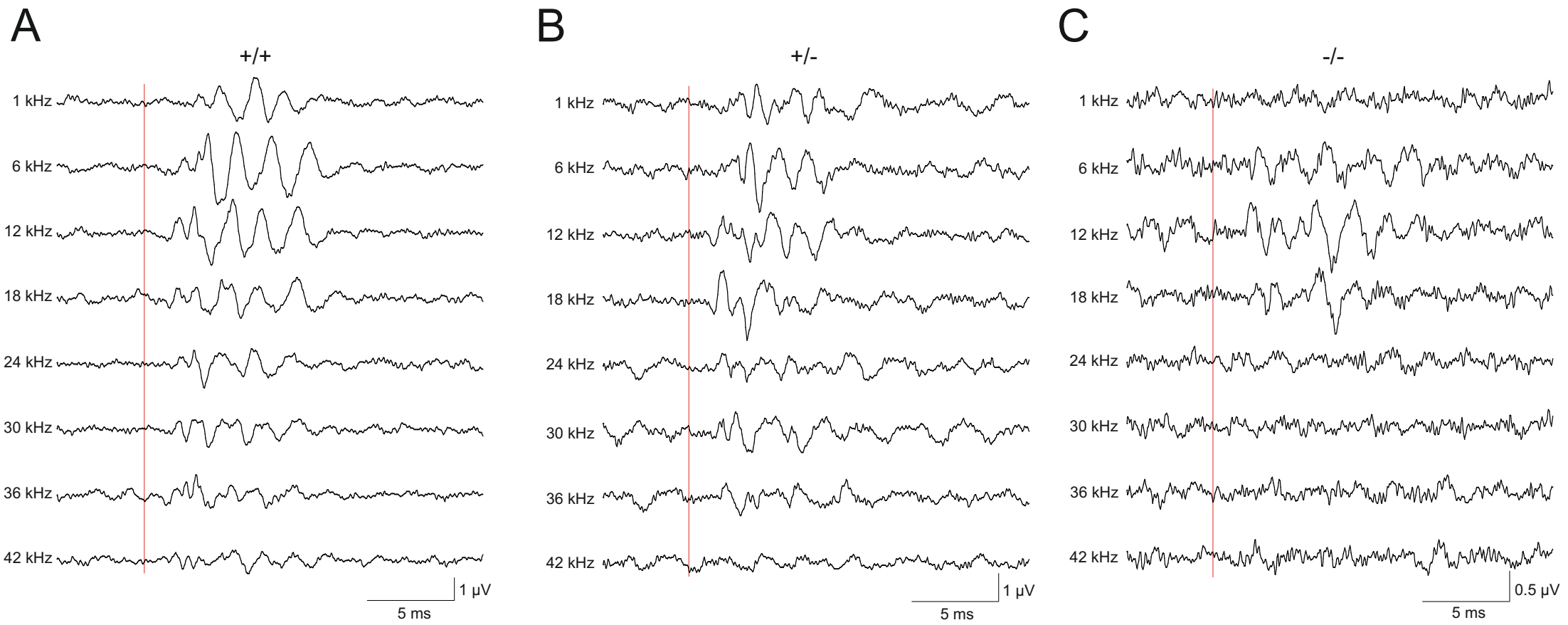

Figure 3
Figure 3 (Lundt et al., 2018)

Click here to access/download;Figure;Figure_3.pdf

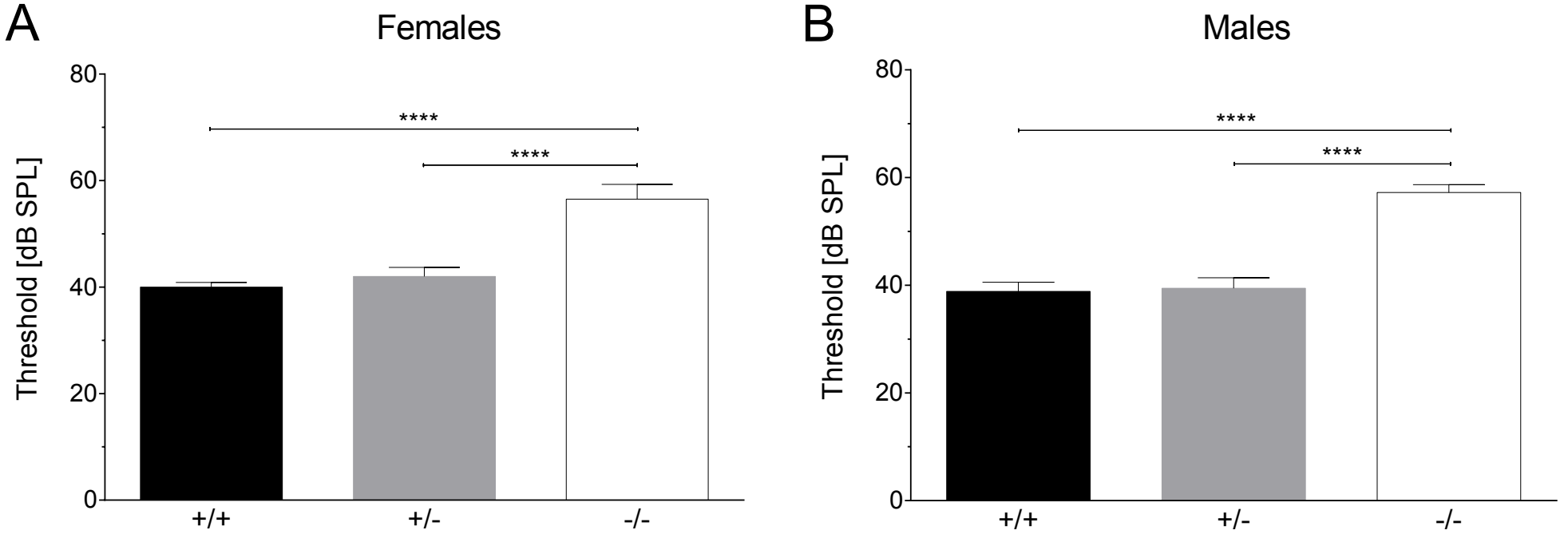

Figure 4
Figure 4 (Lundt et al., 2018)

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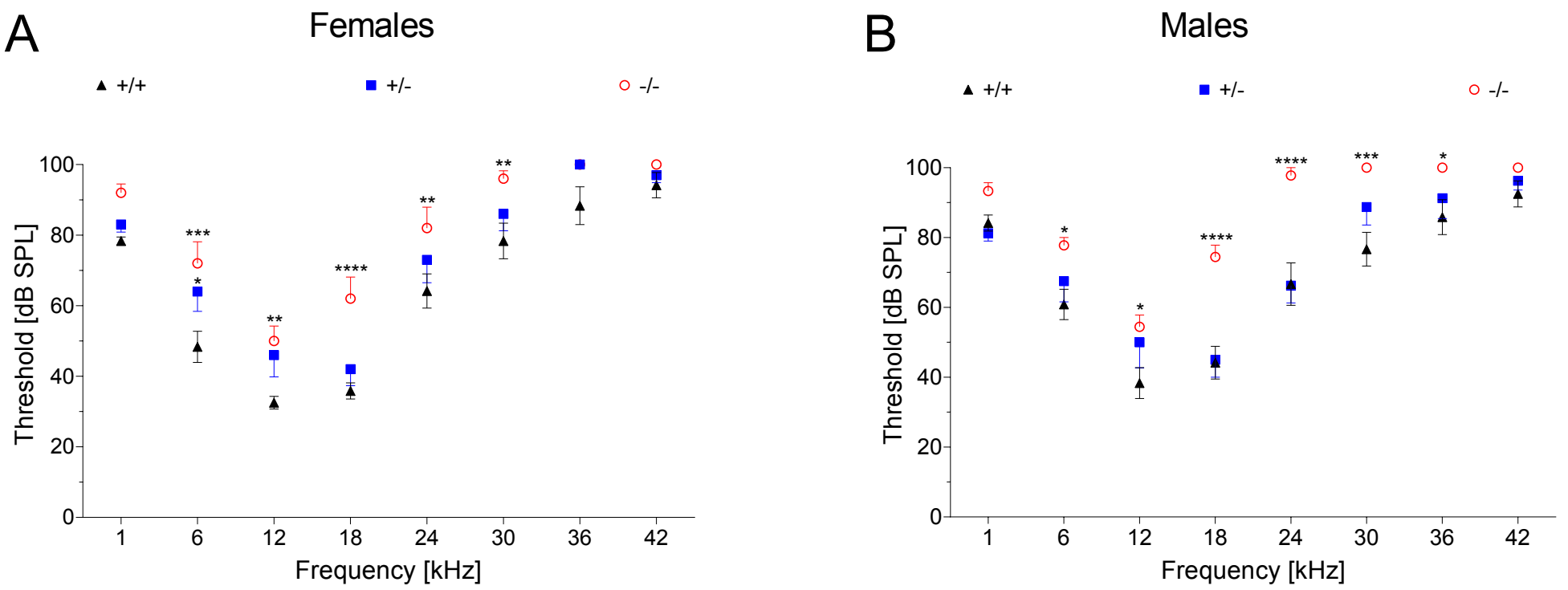

Figure 5 (Lundt et al., 2018)

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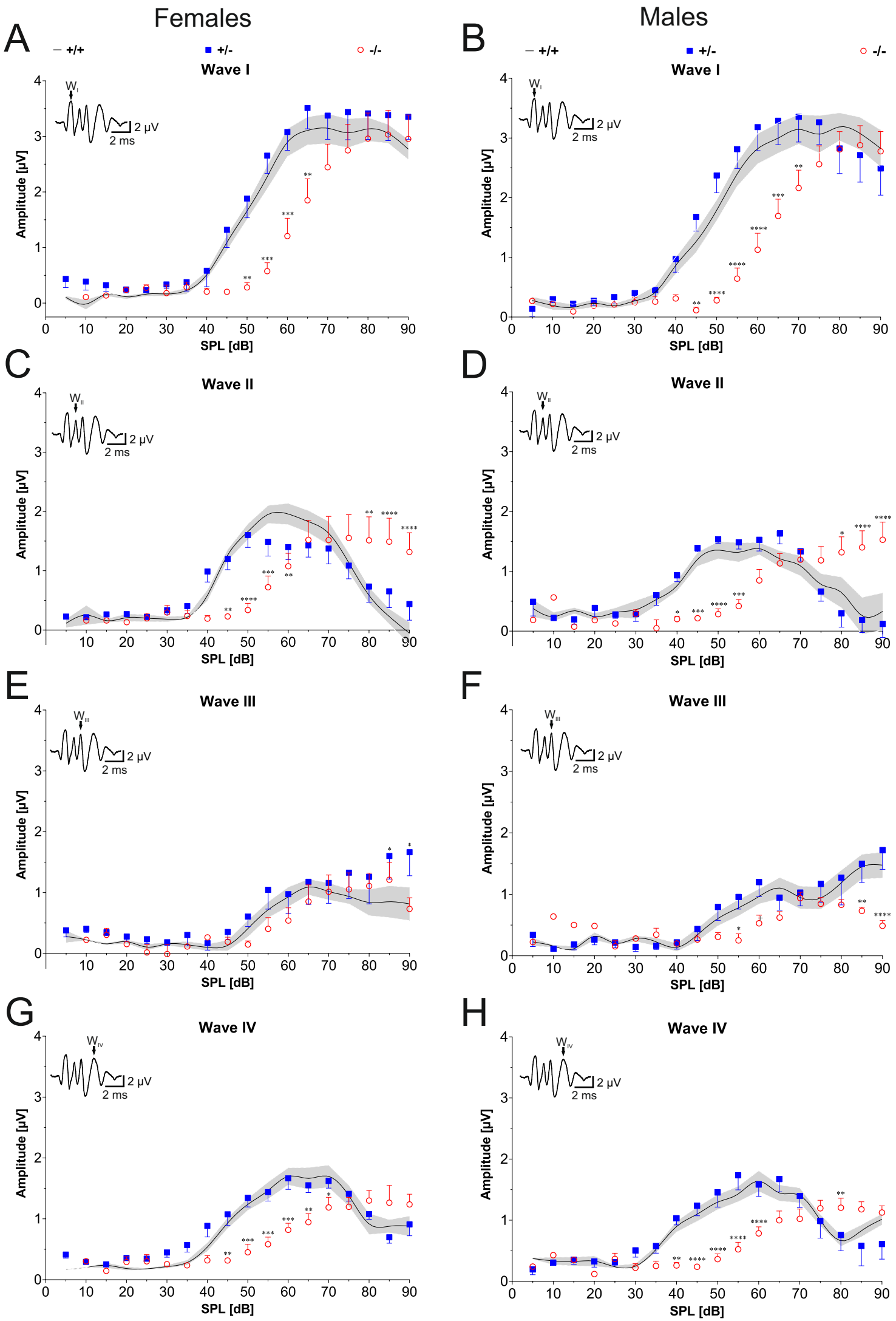

Figure 6
Figure 6 (Lundt et al., 2018)

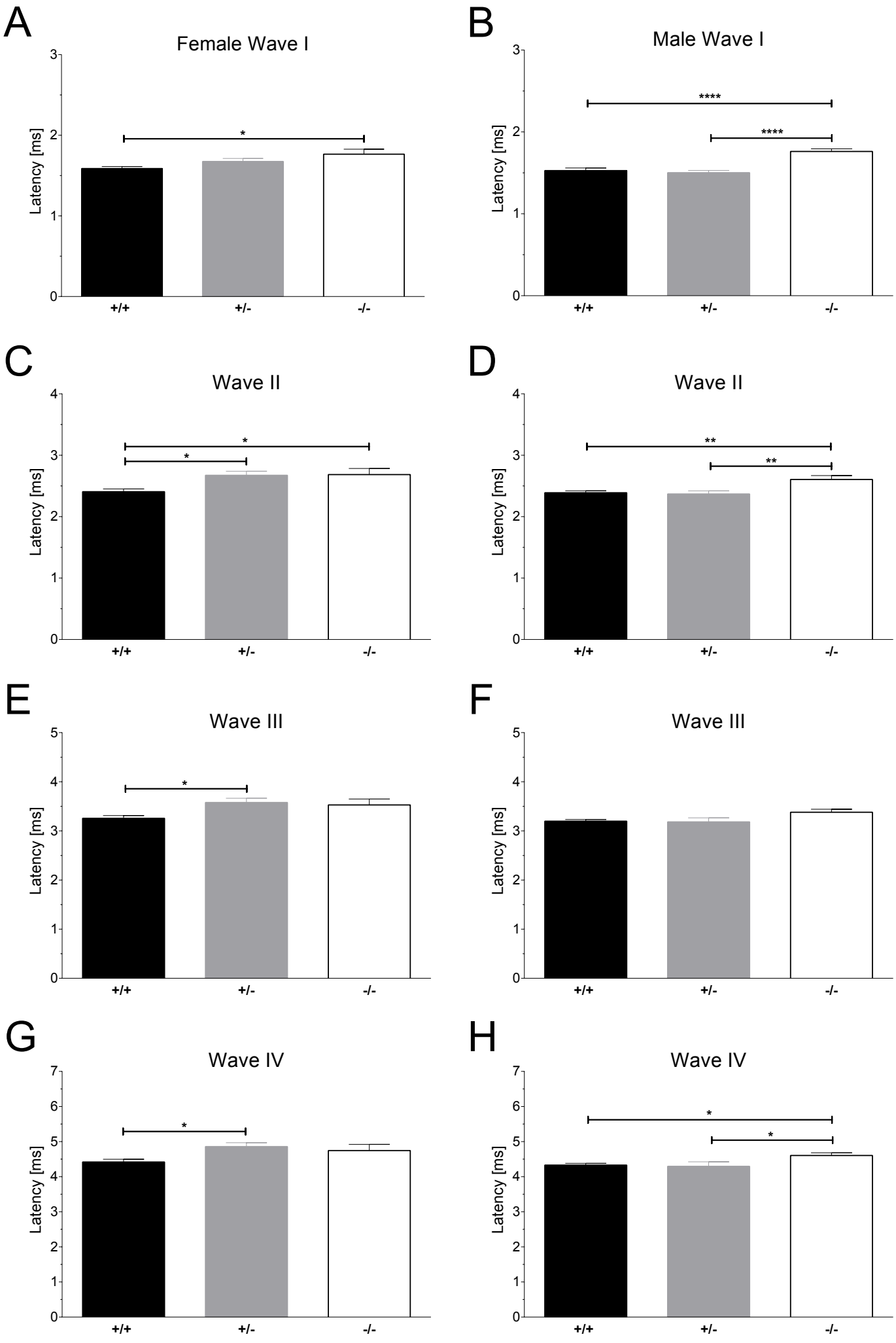

Table of Materials

Click here to access/download;Table of Materials;JoVE_ABR_Materials_final_02102018.xls

Name of Material/ Equipment	Company	Catalog Number
AEP/OAE Software for RZ6 (BioSigRZ software)	Tucker-Davis Technologies (TDT)	BioSigRZ
Binocular surgical magnification microscope	Zeiss Stemi 2000	0000001003877, 4355400000000, 0000001063306, 4170530000000, 4170959255000, 4551820000000, 4170959040000, 4170959050000
Cages (Macrolon)	Techniplast	1264C, 1290D
Carprox vet, 50mg/ml	Virbac Tierarzneimittel GmbH	PZN 11149509
Cold light source	Schott KL2500 LCD	9.705 202
Cotton tip applicators (sterile)	Carl Roth	EH12.1
Custom made meshed metal Faraday cage (stainless steel, 2 mm thickness, 1 cm mesh size)	custom made	custom made
5% Dexpanthenole (Bepanthen eye and nose creme)	Bayer Vital GmbH	PZN: 01578681
Disposable Subdermal stainless steel Needle electrodes, 27GA, 12mm	Rochester Electro-Medical, Inc.	S03366-18
Surgical drape sheets (sterile)	Hartmann	PZN 0366787
Ethanol, 70%	Carl Roth	9065.5
1/4'' Free Field Measure Calibration Mic Kit	Tucker-Davis Technologies (TDT)	PCB-378C0
Gloves (sterile)	Unigloves	1570
Graefe Forceps-curved, serrated	FST	11052-10
GraphPad Prism 6 Software, V6.07	GraphPad Prism Software, Inc.	https://www.graphpad.com/
Heat-based surgical instrument sterilizer	FST	18000-50
Homeothermic heating blanked	ThermoLux	461265 / -67
Ketanest S (Ketamine), 25mg/ml	Pfizer	PZN 08707288
Ringer's solution (sterile)	B.Braun	PZN 01471434
Matlab software	MathWorks, Inc.	https://de.mathworks.com/products/matlab.html
Medusa 4-Channel Low Imped. Headstage	Tucker-Davis Technologies (TDT)	RA4LI
Medusa 4-Channel Pre-Amp/Digitizer	Tucker-Davis Technologies (TDT)	RA4PA
Microphone	PCB Pieztronics	378C01
Multi Field Speaker- Stereo	Tucker-Davis Technologies (TDT)	MF1-S
Oscilloscope	Tektronix	DPO3012
Optical PC1 express card for Optibit Interface)	Tucker-Davis Systems (TDT)	PO5e
Askina Braucel pads (cellulose absorbet pads)	B.Braun	PZN 8473637
Preamplifier	PCB Pieztronics	480C02
RZ6 Multi I/O Processor system (BioSigRZ)	Tucker-Davis Technologies (TDT)	RZ6-A-PI
0.9% saline (NaCl, sterile)	B.Braun	PZN:8609255
SigGenRZ software	Tucker-Davis Technologies (TDT)	https://www.tdt.com/
Software R (version 3.2.1) + Reshape 2 (Version 1.4.1) + ggplot 2 (version 1.0.1) + datatable (version 1.9.4), + gdata (version 2.13.3), + pastecs (version 1.3.18), + waveslim (version 1.7.5), + MassSpecWavelet (version 1.30.0)	The R Foundation, R Core Team 2015	Open Source Software (freely distributable)
Sound attenuating cubicle	Med Associates Inc.	ENV-018V
Standard Pattern Forceps, 12cm and 14.5 cm length	FST	11000-12, 11000-14
Leukosilk tape	BSN medical GmbH & Co. KG	PZN 00397109
Tissue Forceps- 1x2 Teeth 12 cm	FST	11021-12
Uniprotect ventilated cabinet	Bioscape	THF3378
Ventilated cabinet	Tecniplast	9AV125P
Xylazine (Rompun), 2%	Bayer Vital GmbH	PZN 1320422

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Author(s): A. Lundt, J. Soos, C. Henseler, M.J. Arshaad, R. Müller, D. Ehninger, J. Hescheler, A. Sachinidis, K. Broich, C. Wormuth, A. Papazoglou, M. Weiergräber

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We thank the editor for the thorough appraisal of our work. We would like to respond to the comments as follows (all changes highlighted in red):

Editorial comments:

Point 1: The manuscript is formatted by the editor, please retain the same.
Response: We did not make any changes in text format.

Point 2: Please provide the step number for each of the screenshots provided as supplementary files.
Response: We have added the step number to the file names of the screenshots (see also below):

BioSigRZ Acquisition Setup #1_steps 4.6.6 and 4.9 and 5.1.2
BioSigRZ Acquisition Setup #2_steps 4.6.6 and 4.9 and 5.1.2
BioSigRZ Click SigGen Variable Control_steps 4.6.7 and 4.11
BioSigRZ Click Stimulus Setup_steps 4.1 and 4.6.6
BioSigRZ Tone Stimulus Setup_steps 4.1 and 4.6.6
SigGenRZ Click Duration_steps 4.6.1 to 4.6.2
SigGenRZ Click Overview_steps 4.6.1 to 4.6.2
SigGenRZ Click Specs_steps 4.6.1 to 4.6.2
SigGenRZ Tone Overview_steps 4.6.3 to 4.6.5
SigGenRZ Tone Specs_steps 4.6.3 to 4.6.5

Point 3: Since the parent publication is still under revision, we will hold off to the publication of this manuscript upon acceptance until the parent manuscript gets published. We will also need copyright permission to reuse the figures from this publication.
Response: We had addressed this issue in the previous revision / response letter. We included the submitted original article in the reference list. The figures are reprinted / modified from the following manuscript, the status of which is currently “minor revision” in “Neuroscience” (deadline for minor revision is 15th of February so that we assume acceptance around that time):
“$Ca_v3.2$ T-Type Calcium Channels Are Physiologically Mandatory For The Auditory System” by Andreas Lundt, Robin Seidel, Julien Soós, Christina Henseler, Ralf Müller, Maheshwar Bakki, Imran Muhammed Arshaad, Dan Ehninger, Jürgen Hescheler, Agapios Sachinidis, Karl Broich, Carola Wormuth, Anna Papazoglou and Marco Weiergräber.
Once the aforementioned manuscript is accepted, I will get copyright permission from the publisher.

Point 4: Please reword lines 513-514, 519-521, 523.

Response: It is not clear to us why these lines should be rephrased as no explanation was given.

a)If similarity is a problem, please note that “maintaining sternal recumbency” is a medical term that cannot be changed.

We have rephrased lines 513-514 (“6.1 Do not leave an animal unattended until it has regained enough consciousness to maintain sternal recumbency.”) as follows:

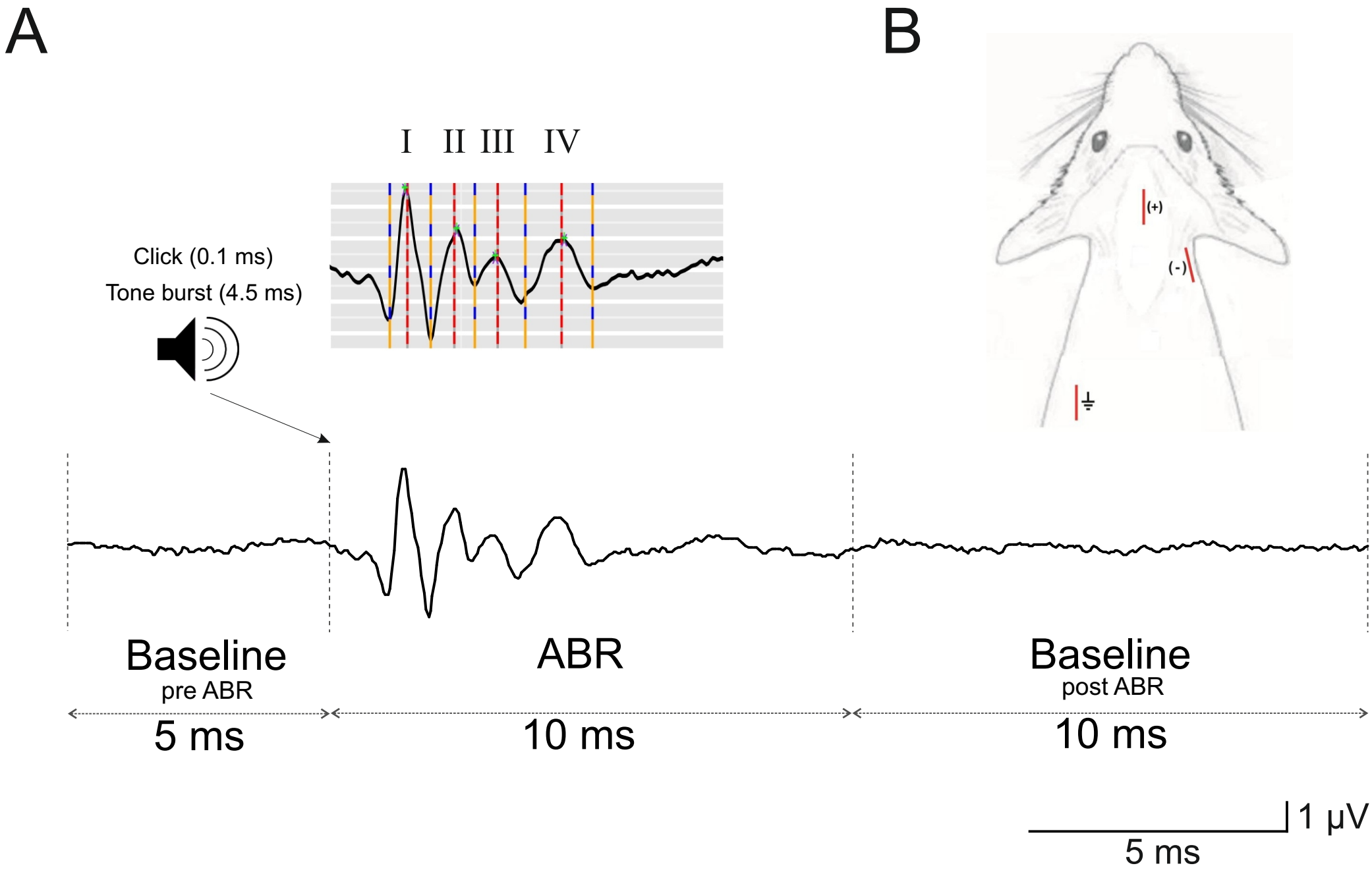
“6.1. Continously monitor the animals until they have regained consciousness and are able to maintain sternal recumbency.”

b) If similarity is a problem here, please note again that we cannot change the dosage description of course. We have slightly rephrased lines 513-514 (“6.3 For post-operative pain management, administer carprofen (mouse: 5 - 10 mg / kg, s.c. once; rat: 2.5 - 5.0 mg / kg, s.c., once).”) as follows:

“6.3) Inject carprofen (mouse: 5 - 10 mg / kg, s.c. once; rat: 2.5 - 5.0 mg / kg, s.c., once) for post-operative pain treatment.”

c) We have rephrased lines 522-523 (“NOTE: As ABR recording electrodes are only inserted subcutaneously, no long-lasting pain management is necessary.”) as follows:

“NOTE: Long-lasting pain treatment is not required as ABR recording electrodes are inserted subcutaneously.”



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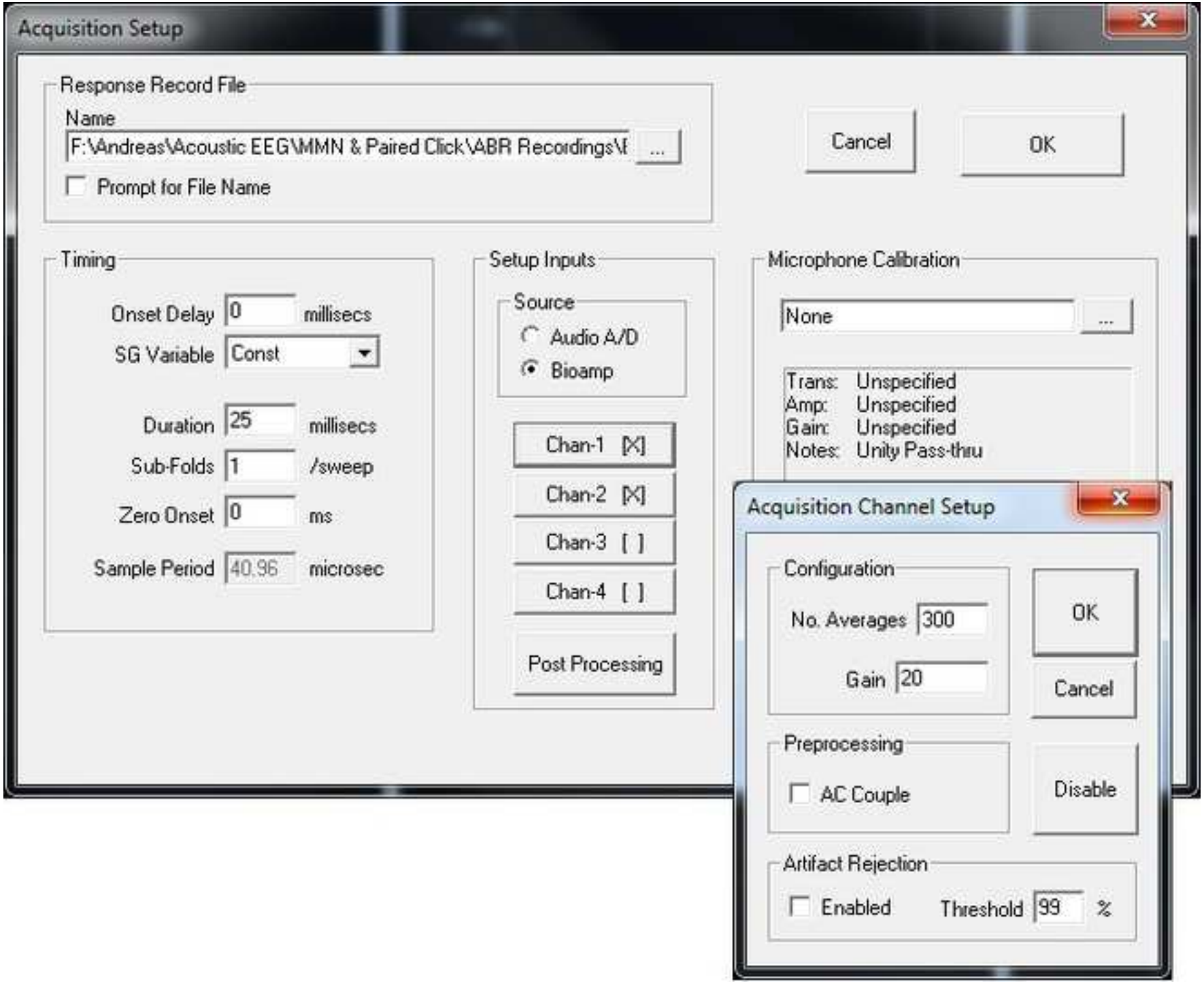

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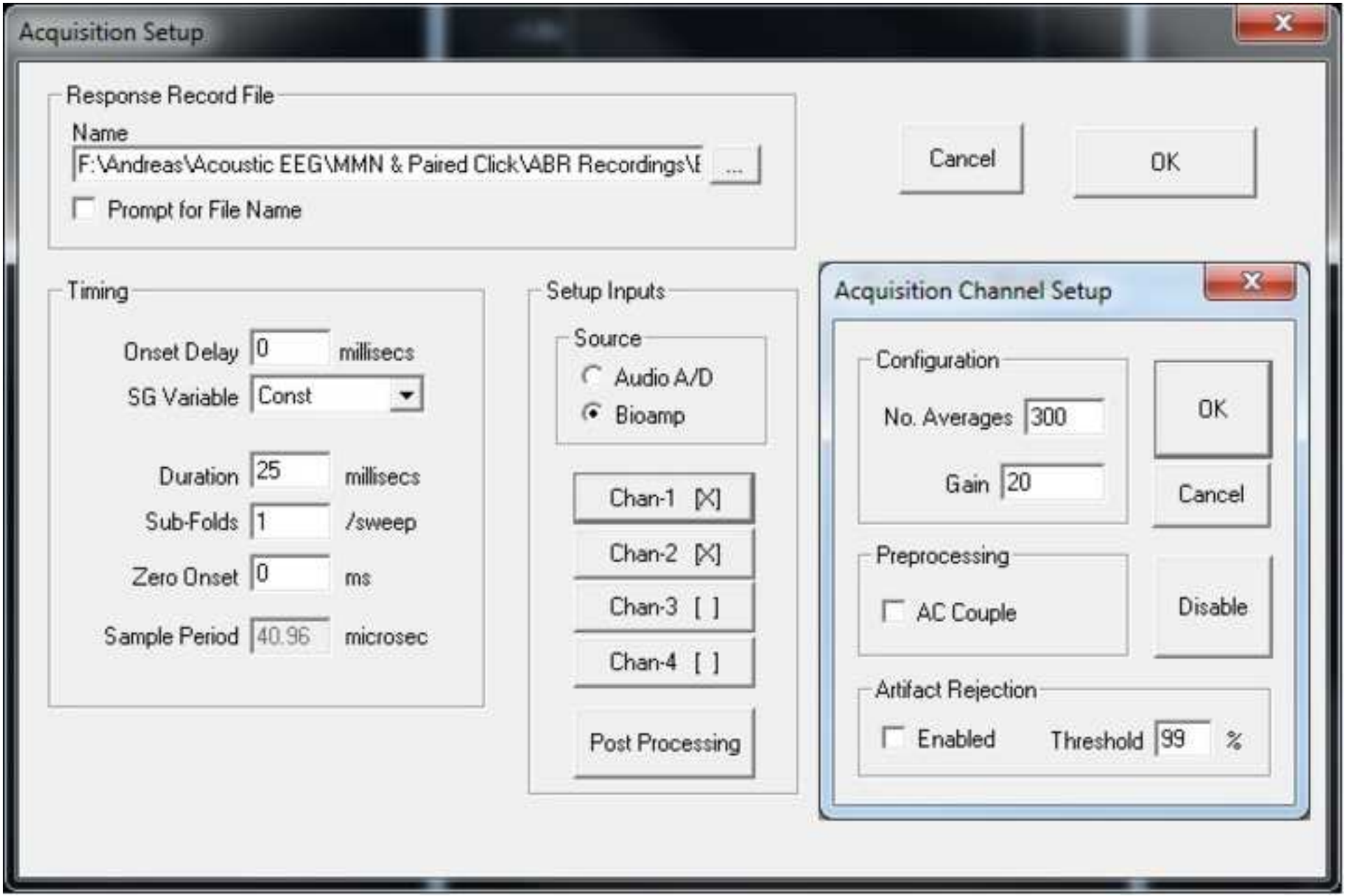

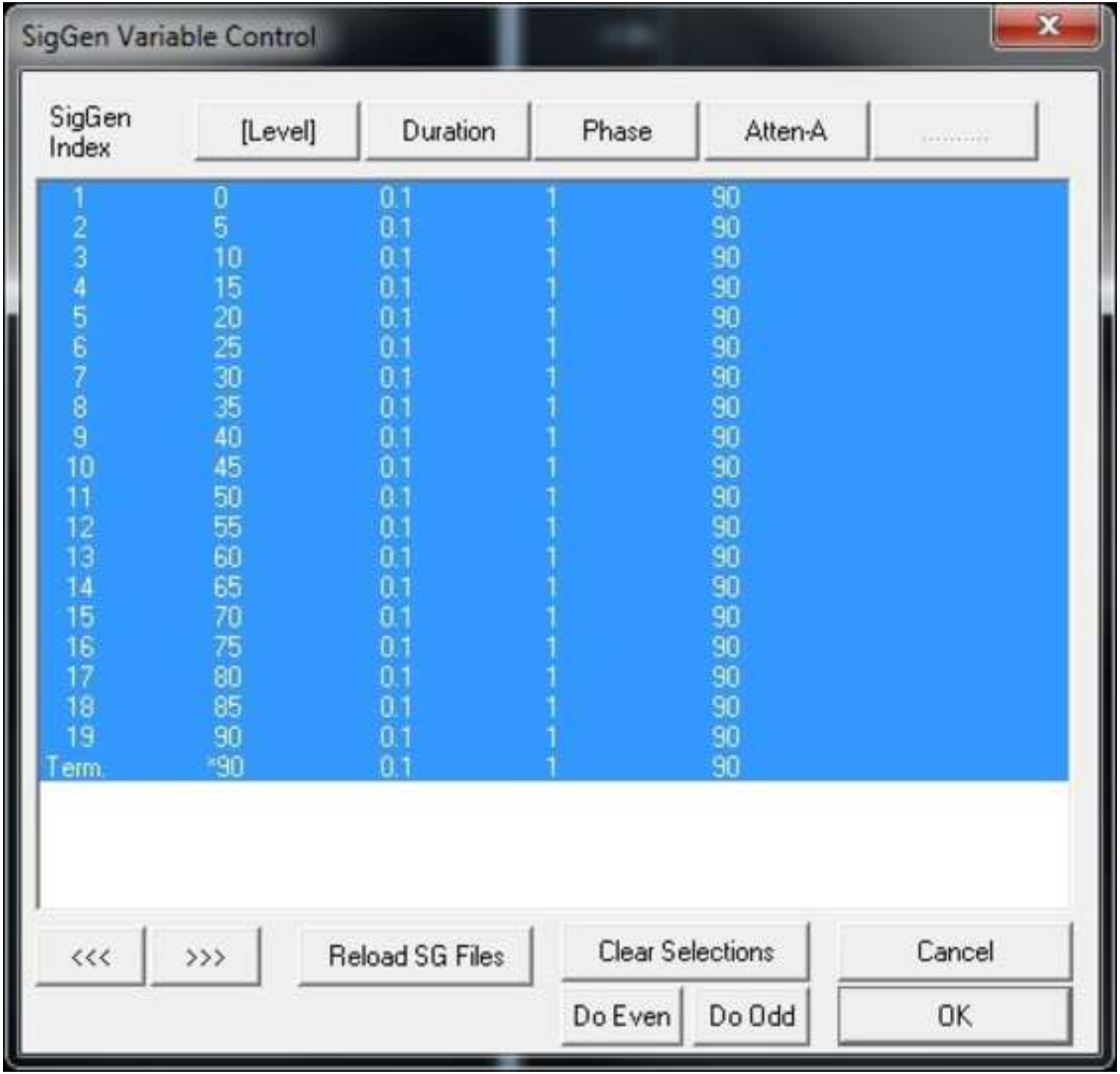

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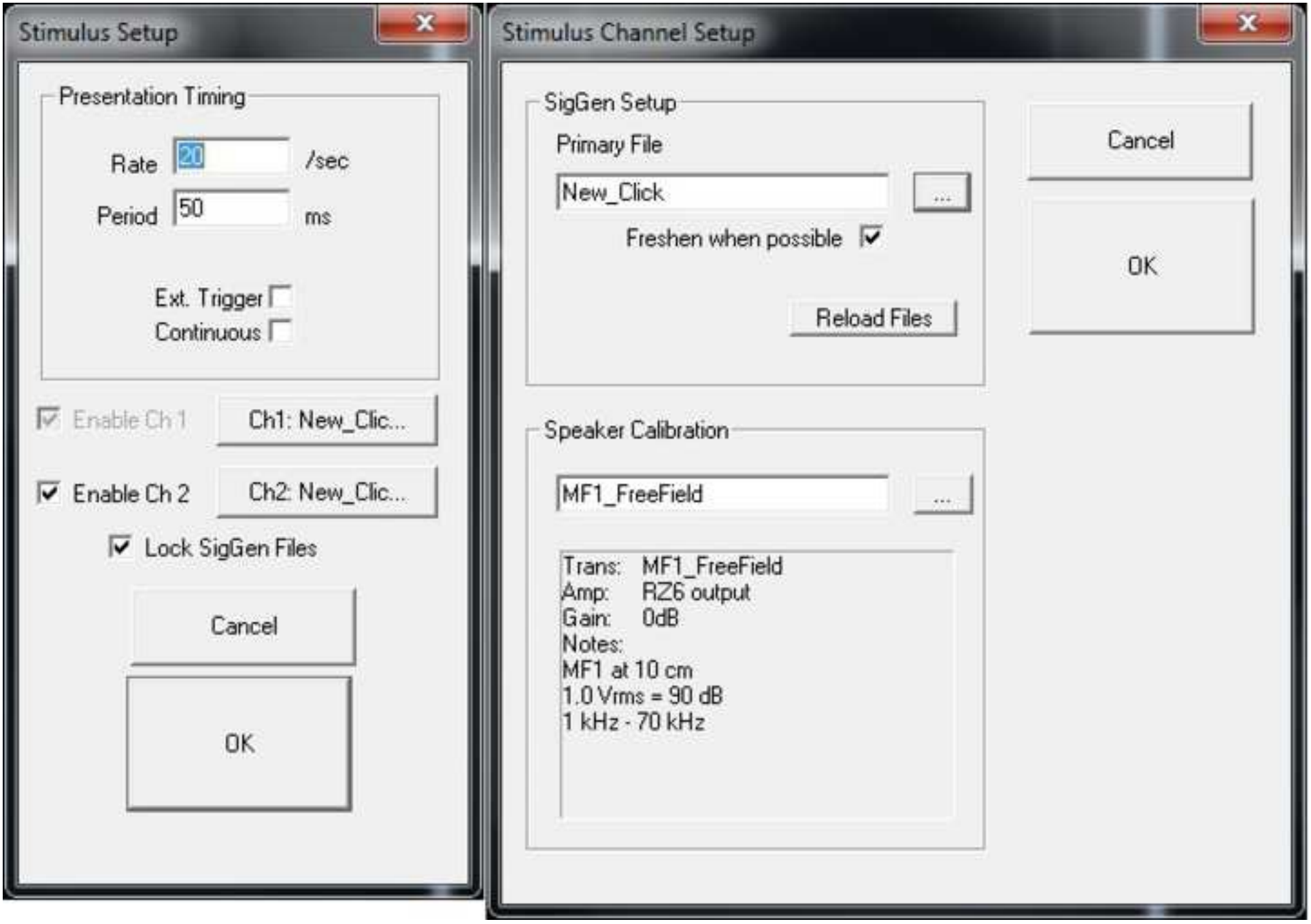

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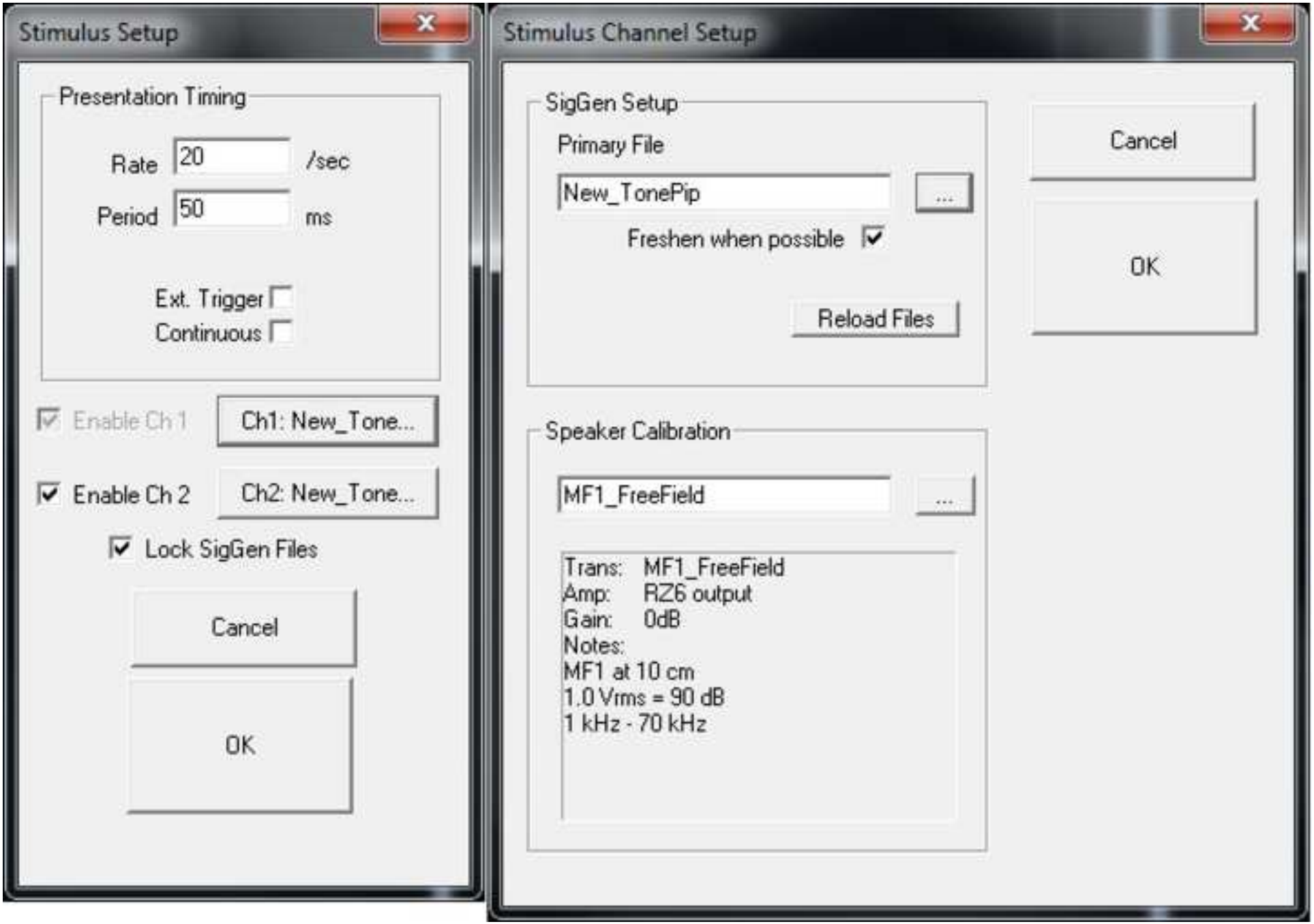

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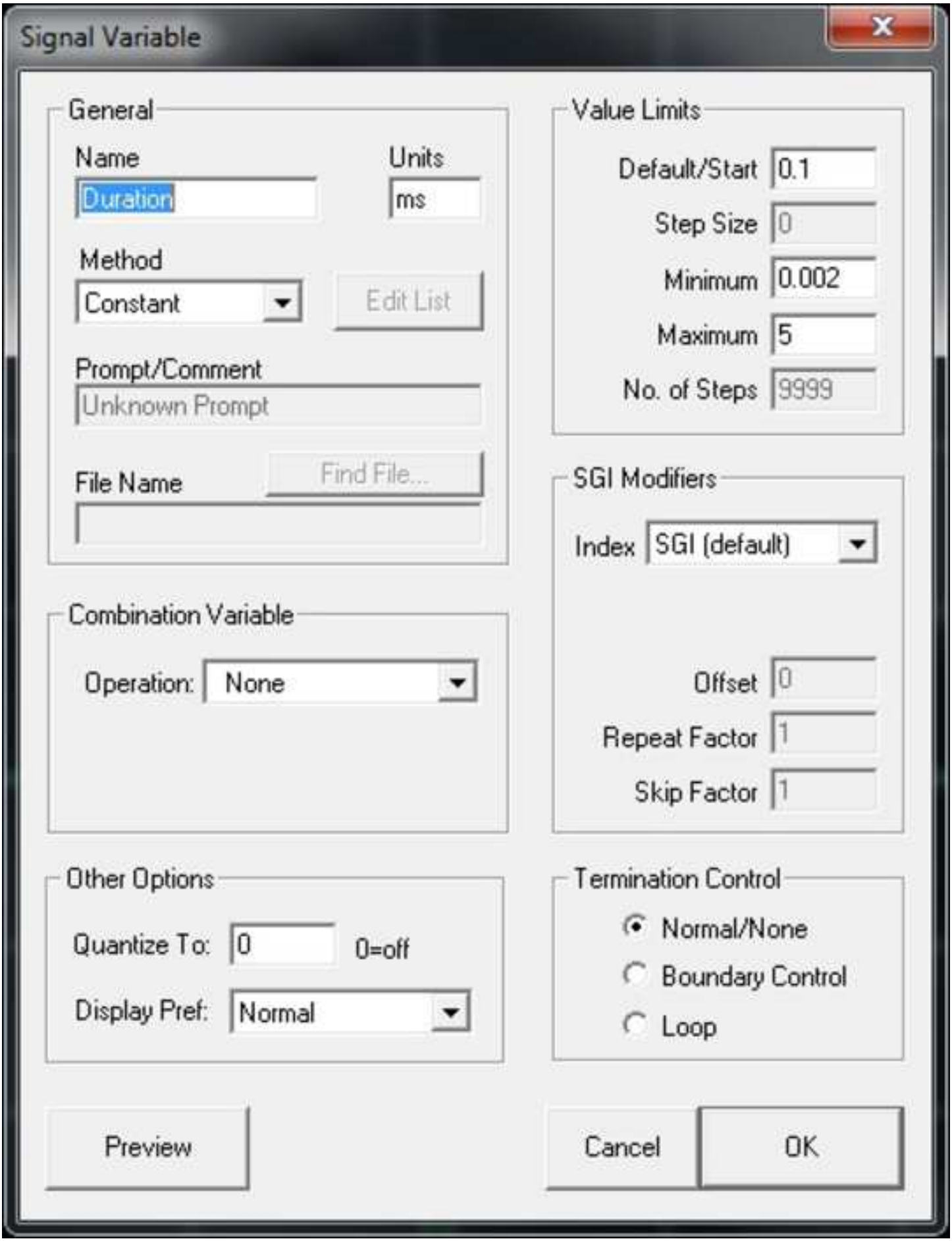

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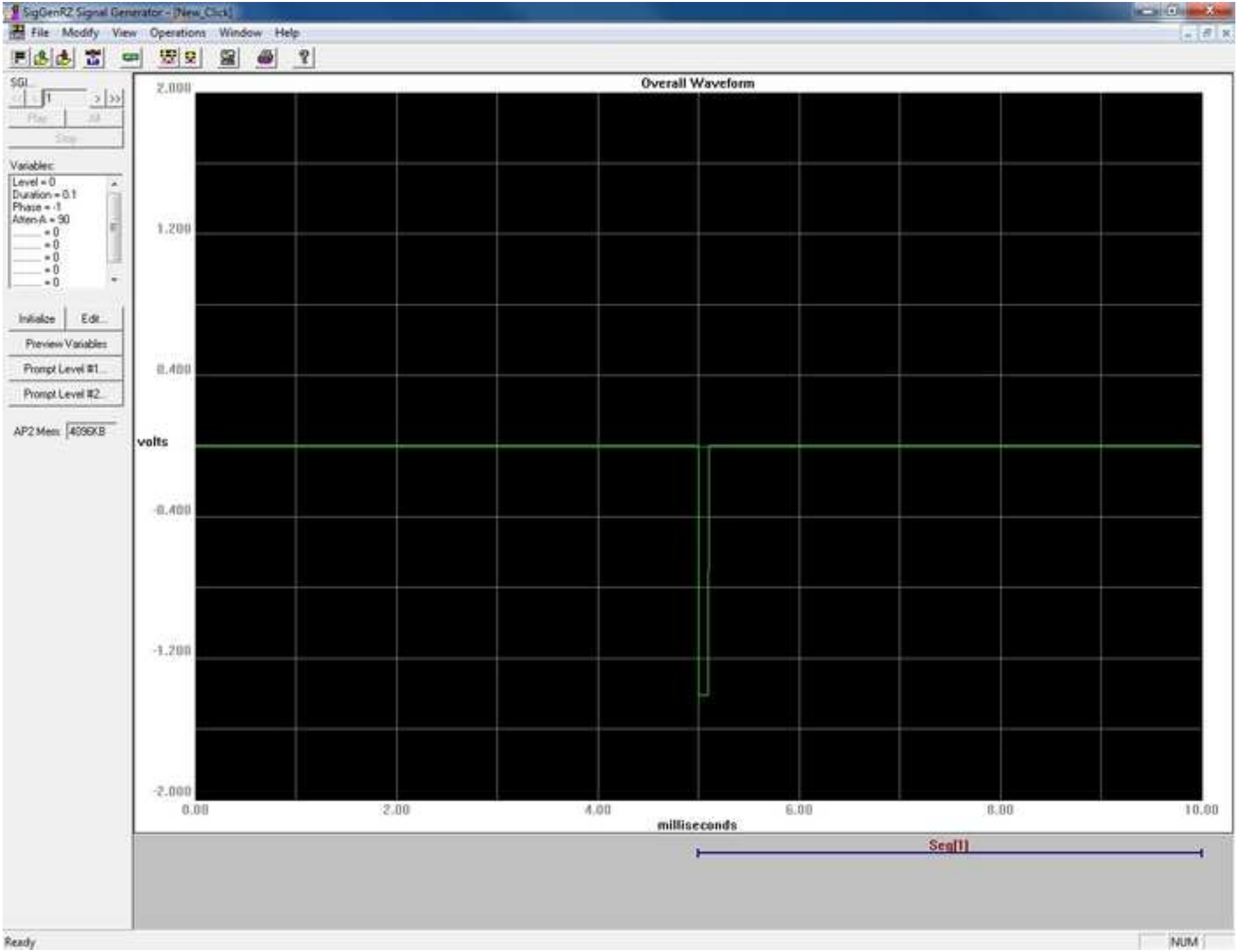

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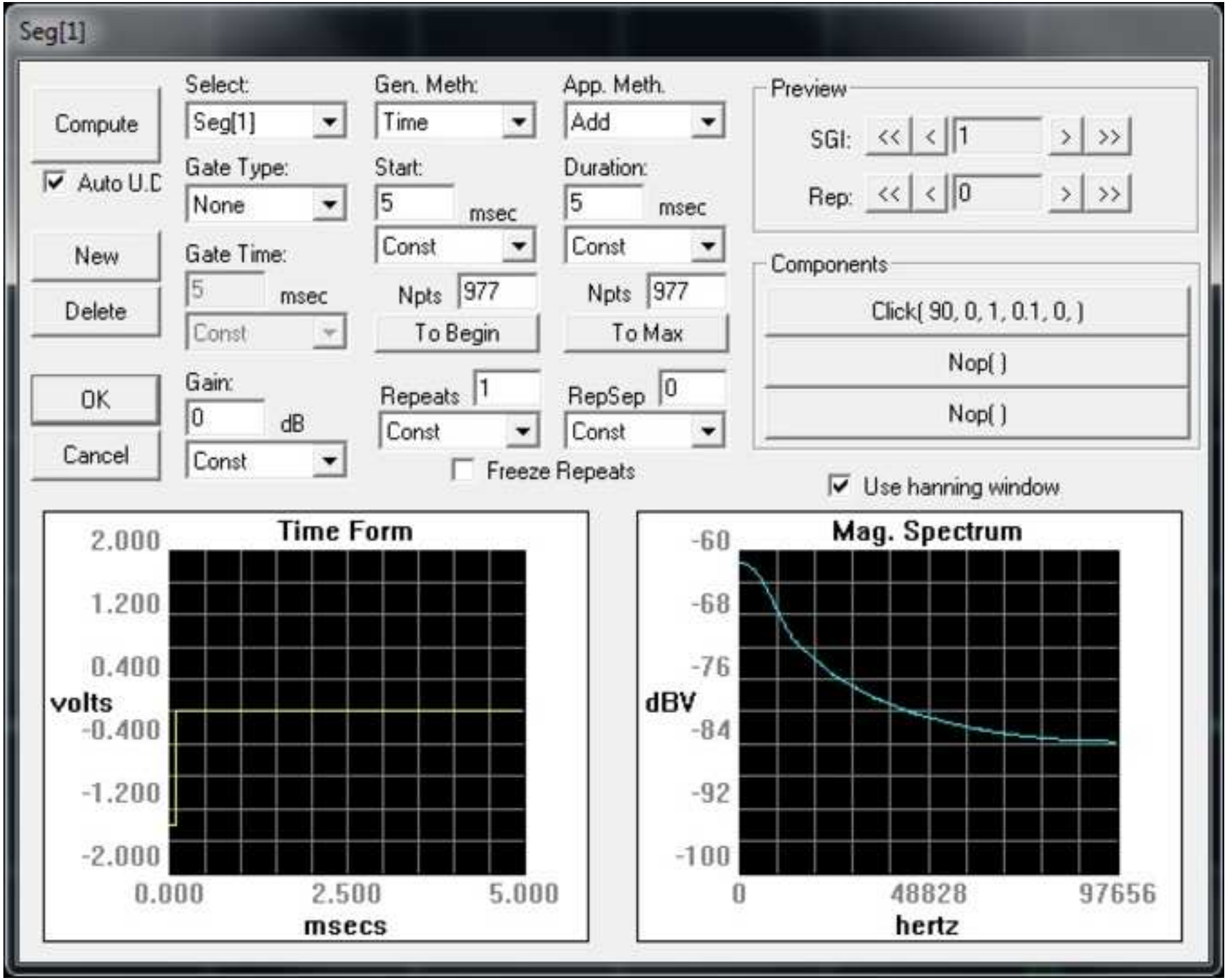

GUI

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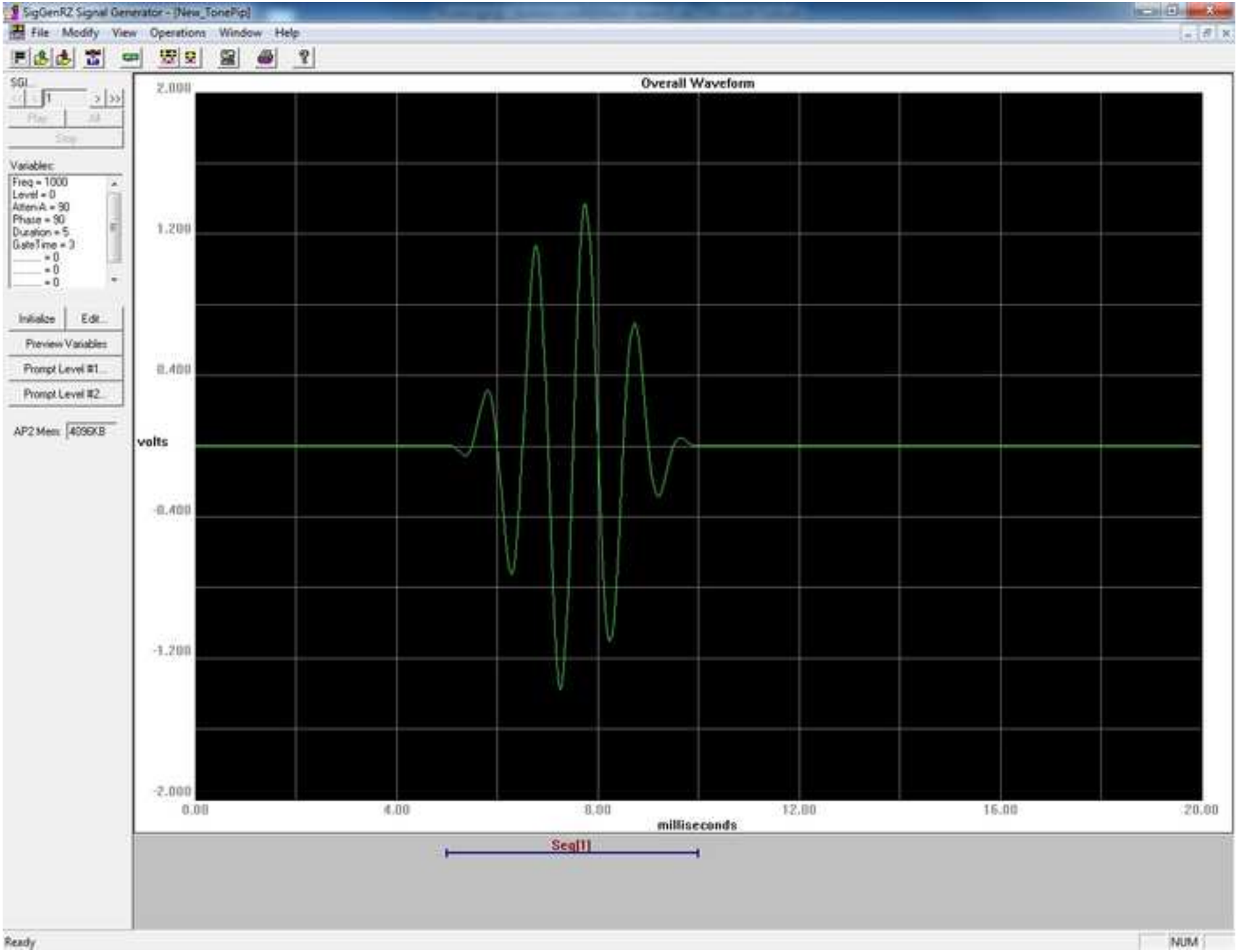

GUI

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