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Auditory and somatosensory pre-pulse inhibition in mice

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Auditory and somatosensory pre-pulse inhibition in mice

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Abstract

Both hearing and somatosensation are sensory responses to vibrations, and here we show a way to investigate such mechanoreceptive psychophysics alone and in combination. Pre-pulse inhibition (PPI) is a well-known, unconditioned, and reflexive technique for measuring sensory thresholds with a wide variety of stimuli and laboratory animals. In this paper, we explore interactions between auditory and somatosensory PPI in normal mice. Fifteen C57/BL6J mice were tested three times each. Ages varied between one and six months. Testing followed published procedures from our lab and others, except the pre-pulses were auditory, somatosensory (vibration of the test chamber), or both. The auditory pre-pulse was an 80 dB SPL broadband noise of 4, 9, 25, or 45 ms duration. Vibrations were of the same duration but different frequencies (500, 460, 360, and 220 Hz respectively). Results show expected auditory responsiveness increasing with duration. There were statistically significant responses to some but not all vibrotactile stimuli. Multimodal responses were approximately additive; the responses to combined auditory and vibratory stimuli were approximately the sum of responses to each stimulus alone (no significant interaction). There is a greater increase with age in the responses to somatosensory than to auditory stimuli. This study provides a behavioral paradigm to assess functional consequences of somatosensory/auditory interactions in mice.

Introduction to the Reader

The following dissertation consists of two main parts. Part I is a manuscript version of the dissertation research. The manuscript was written in the format for anticipated submission for publication in the research journal Physiology and Behavior. Part II is a longer extended literature review and discussion. The extended literature review goes into further detail on auditory and somatosensory pre-pulse inhibition, as well as provides a condensed summary of current research on the development and mechanisms of multimodal sensory interaction within the lateral cortex of the inferior colliculus. The extended discussion includes a more thorough explanation on possible causes and future implications of our results. There is some duplication between Parts I and II, as pieces of the extended literature review and discussion were extracted to form components of the manuscript.

Part I: Manuscript

Auditory and somatosensory pre-pulse inhibition in mice

Anna Louthan, Lincoln Gray, and Mark L. Gabriele

Keywords: acoustic startle response, pre-pulse inhibition, lateral cortex of the inferior colliculus, threshold, vibrotactile, multimodal

I. Introduction

The startle reflex is a motor response to an unexpected, intense stimulus. This experiment used a loud and rapid acoustic stimulus to elicit a startle response; however, tactile, visual, and olfactory stimuli can also elicit a startle response (Koch, 1999). We quantified the startle reflex in mice as the full-body (jerk) of the limbs, but other investigators have used a variety of measures, such as eye-blinks in humans and escape movement in mollusks (Flaten, 2002; Mongeluzi, Hoppe, & Frost, 1998).

A less intense pre-pulse stimulus can attenuate the startle response. A pre-pulse is a stimulus that does not elicit a startle, but if a subject perceives a pre-pulse presented approximately 30-500 ms before the startle-eliciting stimulus, the startle reflex reduces. This is known as pre-pulse inhibition, or PPI. A pre-pulse can be an auditory, somatosensory, or visual stimulus (Koch, 1999). Many diverse animals show PPI, including humans, mice, rats, pigeons, and sea slugs (Mongeluzi, Hoppe, & Frost, 1998; Hoffman & Ison, 1980).

Because of recent findings concerning the auditory and somatosensory afferents into a midbrain structure involved in PPI (the lateral cortex of the inferior colliculus or LCIC) (Balsamo & Gabriele, 2015; Cramer & Gabriele, 2014; Dillingham, Gay, Roxana, & Gabriele, 2017; Wallace, Harris, Brubaker, Klotz, & Gabriele, 2016; Lesicko, Hristova, Maigler, & Llano, 2016), we endeavored to explore multi-modal psychophysical responses that might be mediated by these pathways.

There is very little literature on multimodal pre-pulse inhibition. Brody, Dulawa, Conquet, and Geyer (2004) considered "multimodal PPI" testing in mice as presenting an auditory, visual (light), or somatosensory (puff of air) pre-pulse stimulus before an auditory, visual, or somatosensory startle-eliciting stimulus. To our knowledge, the present study is the first to report vibratory pre-pulse inhibition in mice. This initial report demonstrates that auditory and somatosensory responsiveness, as well as their combinations, can be investigated using PPI in mice. Eventually our goal is to explore how genetic mutations, believed to influence the establishment of LCIC connectivity, might affect behavior.

II. Methods

2.1. Subjects

C57BL/6J mice (n=15) in three age groups including six young (30-67 days old), five middle-aged (108-125 days old), and four old (166-181 days old) were tested three times each. Apart from the one hour testing sessions, mice

constantly had access to food and water, and were housed with their same sex in BioZone Inc. MiniRack™ individually HEPA filtered cages. All procedures were approved by the James Madison University Institutional Animal Care and Use Committee (IACUC).

2.2. Apparatus and stimuli

All testing took place within a 2.13 m x 2.13 m Industrial Acoustic double-walled and double-floored sound-attenuating booth. We used the inside part of a San Diego Instruments SR-Lab Small Animal test chamber (SR Lab: SDI Startle Response System, 2016). The mice were tested in a Plexiglas tube with an inside diameter of 5 cm and a length of 12.5 cm. The tube was glued to a 124 by 200 by 4.5 mm horizontal plate, and glued beneath the plate was an accelerometer. The tube was placed 15 cm directly under a Ross Audio Systems TW 30 compression tweeter, which produced the startle-eliciting stimulus (SES). Figure 1 shows a schematic of the multi-modal testing equipment. A Tucker Davis Technology Real-Time Processor (TDT RP2.1, running at 50 kHz) formed the SES with amplification by a Crown XLS202 amplifier. The SES remained the same for all testing: a 110 dB SPL, 15 ms broadband noise high-pass filtered at 8 kHz with .01 ms linear gate.

Two modalities of pre-pulses were used for testing: somatosensory and auditory. A second Ross TW30 produced the broadband auditory pre-pulse stimuli, and was positioned 38 cm from the long side of the tube (drawn in a different position in Figure 1 so that the speaker can be seen). Four different auditory pre-pulse stimuli were all 80 dB SPL broadband noise, high-pass filtered

at 4 kHz, with instantaneous rise/decay times, of varying durations: 4, 9, 25, or 45 ms.

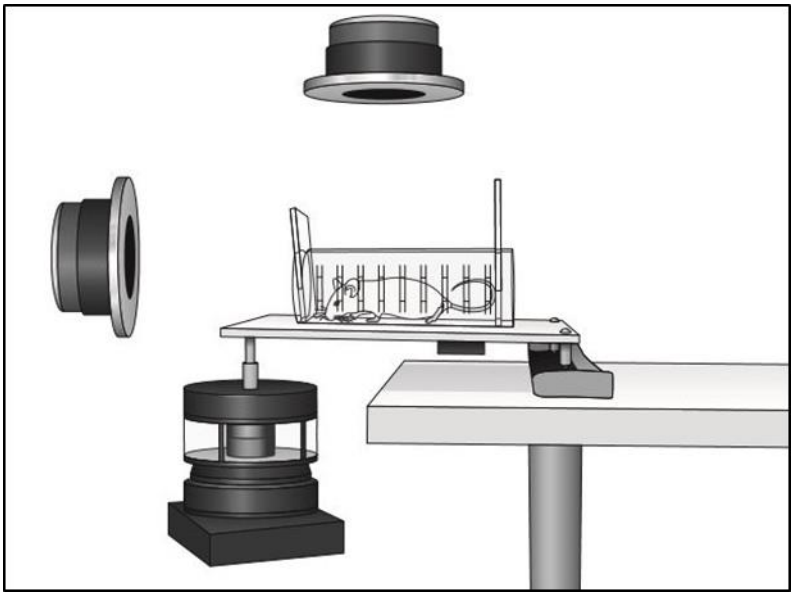

Figure 1. Illustration of the multimodal testing system. The lateral speaker was 90 degrees different in horizontal position than shown, behind the mouse.

The somatosensory stimuli consisted of vibrations of varying frequencies and durations generated with a 1.5V p-p sine wave from an Agilent 33220A Function Generator. This output went directly to a Pasco Mechanical Wave Vibrator (SF-9324). The single, 6 mm diameter piston at the top of the Pasco vibrator was fit into a clip in the middle of one short side of the SR-Lab plate. The two stand-offs on the opposite short side of the plate were placed on foam to reduce sound produced by the vibration. Table 1 describes the four different somatosensory stimuli: 2 cycles of 500 Hz vibration (4 ms long), 4 cycles of 460 Hz vibration (9 ms long), 9 cycles of 360 Hz vibration (25 ms long), and 10 cycles of 220 Hz vibration (45 ms long).

Auditory calibrations used a B&K4939 3/8 inch high-frequency microphone, Listen Inc. SoundConnect Amp and an Agilent 35670A Dynamic Signal Analyzer. The microphone was clamped in the middle of the testing tube during calibration. Calibration of the SES and of the auditory pre-pulses revealed a flat

frequency band from the high-pass limit to 25 kHz, with a gradual high-frequency roll-off.

Vibratory calibrations used the Sensor Kinetics Pro (V 3.1.2) Android app (Sensor Kinetics Pro, n.d.) on an LG-P659 cell phone. The cell phone (139 g) was placed on the SR-Lab test plate beside the tube that typically holds the mouse, and the SR-Lab small-animal test system as well as the LG phone were vibrated by the calibrator supplied with the SR-lab systems; the app records acceleration in (m/s/s) during presentation of the calibrating vibration. RMS voltages recorded from the accelerometer were related to acceleration (in m/s/s or g-force) reported by the Android app (Table 1). Each mV from the accelerometer equals 52 microG or ~0.5m/s/s.

Table 1. The four somatosensory pre-pulses (µG is .0098mm/s/s acceleration).

Duration (ms)	*Cycles*	*Frequency (Hz)*	*ISI (ms)*	*RMS Amplitude (µV)*	*Acceleration (µG)*
4	2	500	200	49	2.5
9	4	460	200	55	2.9
25	9	360	200	118	6.1
45	10	220	150	331	17.2

Sounds produced by vibrations were inaudible to the mice. A Matlab function, seen in Appendix 1 as dB ML, confirmed the air-conducted vibrations from the somatosensory stimulation were below estimated audiometric thresholds of young C57 mice. The four combinations of frequency, duration, and inter-stimulus interval used as the somatosensory stimuli, included a variety of frequencies that produced no audible sound, maximized the peak vibratory

stimulus, and minimized residual vibration persisting during the 100 ms of response recording.

2.3. General procedures

Each mouse was tested three times, with at least one week separating each testing session. Each testing session consisted of 11 blocks with 15 trials each – 165 trials with an inter-trial interval that varied randomly between 15 and 25 s (uniformly distributed). The 15 trials in each block consisted of the following in random order:

- four trials, one with each somatosensory pre-pulse followed by the SES
- four trials, one with each auditory pre-pulse followed by the SES
- four trials with a simultaneous somatosensory and auditory pre-pulse of identical length (4ms vibration with the 4ms sound … 45ms vibration with 45ms sound) followed by the SES
- two control trials in which the SES was presented alone
- one trial with no pre-pulse or SES

For each trial, RMS voltage from the accelerometer was recorded for 100 ms from the start of the SES. Pre-pulse inhibition was calculated as 1 – (ASR_p)/(ASR_c) as in Allen and Ison (2010). In this equation, ASR_p represents the acoustic startle response in the pre-pulse stimulus conditions. ASR_c represents the acoustic startle amplitude in the control condition, without a pre-pulse stimulus before the SES. The PPI is thus the reduction in startle amplitude when

a pre-pulse was present; larger positive fractions represent greater PPI, and a value of 0 represents the absence of PPI.

III. Results

An initial repeated-measures ANOVA included test-number (the first, second, and third test of each mouse) along with PPIs to 12 stimuli as within-subjects variables and group (three ages) as the between-subjects variable. There was no significant main effect nor interaction with test-number (p's > 0.7). Therefore, the three tests of each mouse were pooled to simplify the analysis (12 PPIs to four durations of three modalities averaged over three tests of 11 blocks).

The important repeated-measures analysis had modality (with three levels: tone alone, vibration alone, or combined) and duration (with four levels: 4, 9, 25 and 45 ms) as within-subjects factors, and age (with three levels: young, medium, and old) as the single between-subjects factor. All within-subjects factors met Mauchly's test of sphericity.

Results showed a significant effect of modality ($F_{2,24}$= 43.3, p< .001, $_p\eta^2$=.78) and a significant modality by age interaction ($F_{4,24}$= 4.6, p= .007, $_p\eta^2$=.43). There was a significant effect of duration ($F_{3,36}$= 23.2, p< .001, $_p\eta^2$=.66), and no duration by age interaction (p=.16), but a duration-by-modality interaction was found ($F_{6,72}$= 6.9, p<.007, $_p\eta^2$=.37). No duration by group interaction (p = .159) and no three-way interaction (p = 0.24) was present. The effect of age was not significant; however, it approaches significance (p = .08, $_p\eta^2$=.34). Figure 2 displays the mean PPI of all mice for the auditory, somatosensory, and combined

auditory/somatosensory pre-pulse presentation at each stimulus duration, 4, 9, 25, and 45 ms. As stated above, each vibration was a different frequency: 500, 460, 360 and 220 Hz, respectively. Responsiveness to the auditory pre-pulses increased as the stimulus duration increased, as expected. Only two somatosensory stimuli, 2 cycles of 500 Hz (4 ms) and 9 cycles of 360 Hz (25 ms), elicited reliable PPI ($p<.05$ in single-sample t-tests comparing the PPI to zero). The results also suggest that the 25 ms broadband sound with 9 cycles of 360 Hz vibration resulted in an additive-like effect of multimodal PPI.

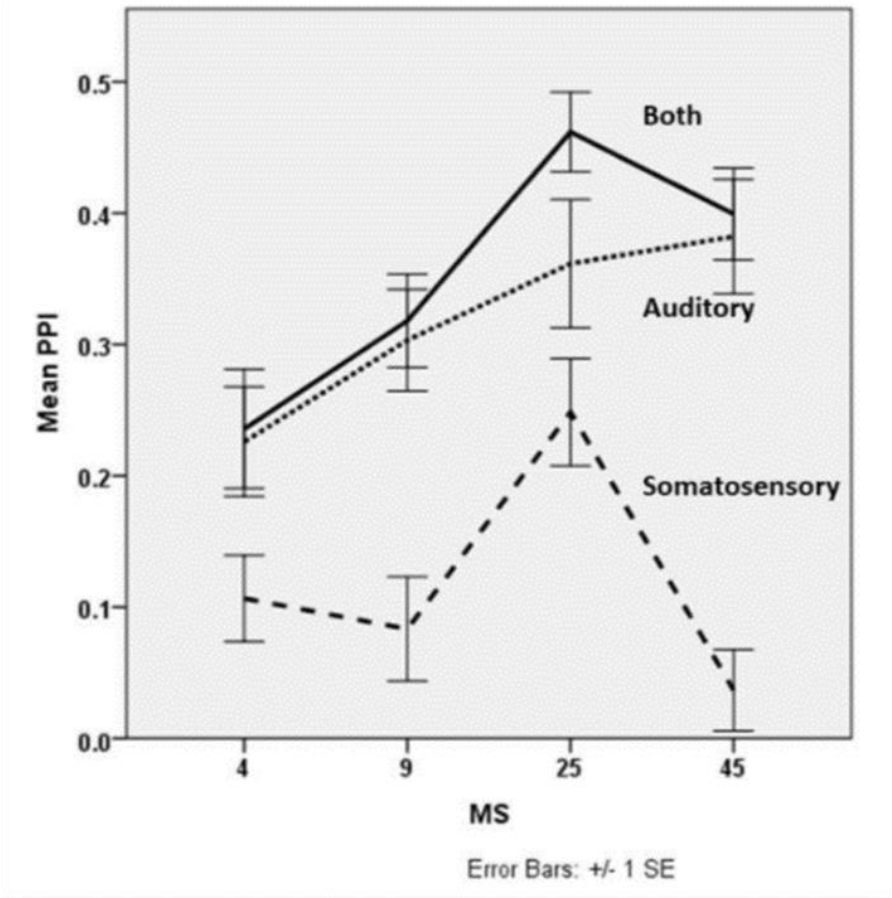

Figure 2. Mean PPI of all mice for the auditory (small dashed), somatosensory (large dashed), and auditory and somatosensory (solid) pre-pulse presentation at each stimulus duration (4, 9, 25, and 45 ms).

Figure 3 displays the PPI trends over the three age groups for the 25 ms pre-pulses alone. The 25 ms stimuli were selected for this analysis because the somatosensory responses were the most robust at that duration. Young mice

had no significant PPI to the vibrations; thus, the multimodal additive effect of PPI is seen in middle-aged and old mice only.

IV. Discussion

It is well established that PPI can be recorded using an auditory pre-pulse in mice (Hoffman & Ison, 1980; Koch, 1999; Fitch, Threlkeld, McClure, & Peiffer, 2008; Liuzzo, Gray, Wallace, & Gabriele, 2014; Parisi & Ison, 1981). The present work shows that somatosensory as well as auditory stimuli, alone and in combination, can elicit PPI in mice.

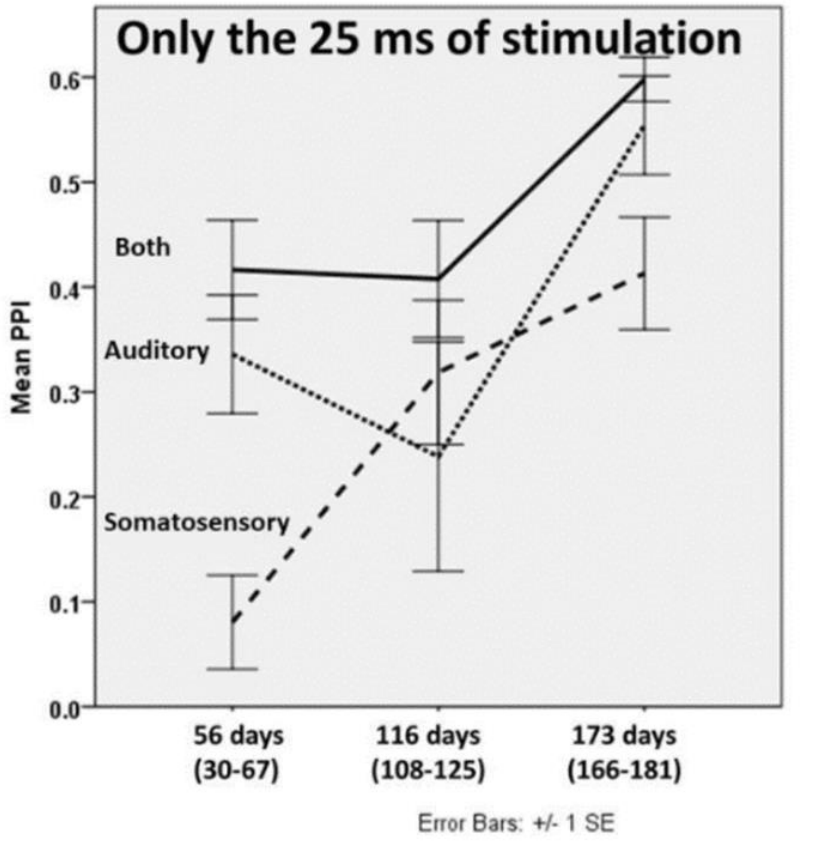

Figure 3. PPI trends with age (young, middle, and old) for the 25 ms broadband auditory pre-pulse, 9 cycles of 360 Hz somatosensory pre-pulse, and both pre-pulses simultaneously.

Reports of somatosensory PPI exist in other species. For example, Mongeluzi, Hoppe, and Frost (1998) found that the marine mollusk, Tritonia diomedea, showed inhibition to a tail shock when a 100 ms vibration was used as a pre-pulse. Pre-pulse inhibition of the acoustic startle response has been found in rats using somatosensory stimulation in the form of electric shock (Pinckney,

1976). Researchers have used somatosensory stimuli as the startle-eliciting response in mice; Brody, Dulawa, Conquet, and Geyer (2004) used an airpuff as the SES in mice with an auditory or visual pre-pulse. To our knowledge, the present findings are the first to report vibratory pre-pulse inhibition in mice.

Each modality affects PPI differently. This is not surprising because there was no attempt to equate the salience of the auditory and somatosensory stimuli, and the combination would likely be different than each modality separately: additive, synergistic, or antagonistic. Each modality's effect on PPI is different across each age group, i.e. not parallel, most evident in Figure 3.

Young mice (~1-2 mo) were less responsive to vibrations than older mice (~3.5-6 mo). While mice at 108 days and older responded to the somatosensory pre-pulse, the young mice at 67 days and younger did not. This finding suggests the possibility of a heightened somatosensory awareness with increased age, potentially co-occurring with decline in hearing. Mammalian tactile sensation can take weeks or months to mature after birth. In cats, tactile receptors and sensory fiber myelination likely do not reach maturity until one to two months of age, and central pathways may not mature until two to three months of age. At vibratory frequencies above 100 Hz, neonatal response thresholds are five to ten times that of adult cats (Rowe, 1982). No information was found on development of somatosensory afferents in mice.

C57BL/6J mice have progressive age-related hearing loss that starts at approximately two months. This loss begins at high frequencies, and then includes middle and low frequencies as the animal ages (Li & Borg, 1991). The

broadband auditory stimuli presented in this experiment certainly include frequencies affected by the early high-frequency hearing loss in middle- and old-aged groups. Evidence for a possible increased representation of the somatosensory system with auditory deprivation, by way of increased trigeminal projections to the cochlear nucleus, is provided by Shore, et al. (2007). They found that guinea pigs with noise-damaged auditory systems had reduced thresholds, decreased latencies, and enhanced response amplitudes to trigeminal (somatosensory) stimulation, and increased numbers of cells with enhanced bimodal integration in the dorsal cochlear nucleus.

Pre-pulse inhibition as a general response may mature with age, possibly explaining the generally positive slopes in Figure 3. Dean, Sheets, Crofton, and Reiter (1990) showed that increasing age, up to postnatal day 35-37, resulted in enhanced auditory gap-detection PPI in rats. Mice are considered adults at 9 weeks (Kempermann, Hg, & Gage, 1997). It may be possible that some or all of the young mice, at 30 – 67 days old, had immature PPI pathways during testing.

Somatosensory PPI was only seen with the 360 Hz and 500 Hz vibrotactile stimuli, but responses at 500 Hz were barely significant. The middle- and old-aged mice were most responsive to 360 Hz vibrations, and not as responsive to the higher and lower frequencies, suggesting a U-shaped curve for vibratory thresholds as a function of frequency. In humans, thresholds of vibration are different across frequencies (Verrillo, 1980).

The pre-pulse combining 9 cycles of 360 Hz vibration with 25 ms broadband sound resulted in an additive-like effect (figure 3), in that the simultaneously

multimodal PPI was close to the sum of the PPI to each modality alone. This behavioral finding supports the notion that auditory and somatosensory afferent pathways converge and thereby influence responsiveness to startling stimuli. The goal of this experiment is to demonstrate multimodal pre-pulse inhibition, and thus to provide a behavioral component for the convergence and crosstalk between somatosensory and auditory systems, as has been described in the lateral cortex of the inferior colliculus (LCIC) (Lamb-Echegaray, Gay, Noftz, & Gabriele, 2018; Cramer & Gabriele, 2014; Dillingham, Gay, Roxana, & Gabriele, 2017; Lesicko, Hristova, Maigler, & Llano, 2016; Wallace, Harris, Brubaker, Klotz, & Gabriele, 2016).

PPI of the acoustic startle reflex by a variety of sensory modalities (auditory, visual, or somatosensory) involves midbrain connections (superior and inferior colliculus) to the pedunculopontine tegmental nucleus (PPTN), which in turn inhibits the acoustic startle reflex. Auditory pre-pulse information projects to the superior colliculus through the inferior colliculus (Fitch, Threlkeld, McClure, & Peiffer, 2008; Koch, 1999). The inferior colliculus, both its lateral and dorsal cortices, are the structures of the acoustic startle-response pathway where the pre-pulse is thought to have its inhibitory effect on the startle-response (Liuzzo, Gray, Wallace, & Gabriele, 2014; Parham & Willott, 1990).

Somatosensory afferents also project to LCIC (Aitkin, Dickhaus, Schult, & Zimmermann, 1978). In multiple mammals, somatosensory inputs from the spinal cord, dorsal column nuclei, spinal trigeminal nuclei, and somatosensory cortex project to the LCIC (Gruters & Groh, 2012; Loftus, Malmierca, Bishop, &

Oliver, 2008; Lesicko, Hristova, Maigler, & Llano, 2016). Recent research has focused on the mechanisms and development of these multimodal projection pathways within the LCIC. The LCIC is layered and receives multimodal inputs that terminate in discretely organized modular and extramodular zones. Converging somatosensory inputs preferentially target discontinuous modular fields that span LCIC layer 2, while auditory afferents terminate in surrounding extramodular domains (Cramer & Gabriele, 2014; Dillingham, Gay, Roxana, & Gabriele, 2017; Wallace, Harris, Brubaker, Klotz, & Gabriele, 2016; Lesicko, Hristova, Maigler, & Llano, 2016; Lamb-Echegaray, Gay, Noftz, & Gabriele, 2018; Lesicko & Llano, 2016). Furthermore, LCIC modular and extramodular zones exhibit cross-talk and influence each other. Thus, it is likely that the LCIC is important for the integration of auditory and somatosensory information (Aitkin, Dickhaus, Schult, & Zimmermann, 1978); however, the functional consequences of these multimodal interactions are not fully understood; hence this pilot study.

In conclusion, this paper describes a behavioral paradigm to assess functional consequences of somatosensory/auditory interactions in mice.

Implication for future research

- Ongoing research in our lab on the anatomical development of these multimodal afferent projections should provide insights concerning current behavioral findings.
- Further research on age trends in somatosensory PPI is warranted.
- Equal response curves to vibrotactile stimuli, similar to those found for humans in Verrillo (1980), could be constructed. Estimates of dB above

thresholds, like those for mouse hearing in the Matlab function dB ML, only for vibrotaction, would be helpful to equate the saliences of multimodal stimuli.

- Our continued experimentation exploring how Eph-ephrin mutations might compromise LCIC multimodal circuit assembly and thus neural processing should inform hypotheses regarding expected differences in PPI in Eph-epherin mutants relative to controls.
- PPI is more effectively elicited by a gap in background noise than by the onset of a sound (Liuzzo, Gray, Wallace, & Gabriele, 2014; Allen & Ison, 2010). Interestingly, gap detection is used as an animal and human model for tinnitus testing (Berger, Coomber, Shackleton, Palmer, & Wallace, 2013; Fournier & Hébert, 2013; Longenecker, Chonko, Maricich, & Galazyuk, 2014; Turner, et al., 2006). Furthermore, somatosensory influence to the cochlear nucleus is heightened after auditory loss. This phenomenon has been speculated to be a mechanism causing tinnitus; therefore, somatosensory-based tinnitus treatments have been developed, but require further investigation (Dehmel, Cui, & Shore, 2008). The procedures described in this report may be utilized to set up an animal model to study possible cross-modal, auditory-somatosensory therapies for tinnitus.

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Part II: Extended Literature Review and Discussion

I. Extended Literature Review

This research project provides the behavioral component to the current neuroanatomical research of the Gabriele laboratory (JMU Biology department) on the multimodal sensory organization of the lateral cortex of the inferior colliculus. Somatosensory and multimodal pre-pulse inhibition in normal mice, two concepts with very little previous research, were explored to gather information pertaining to the behavioral functions of a normal sensory system's response to multimodal sensory stimulation.

1.1. The unconditioned startle reflex and pre-pulse inhibition

The startle reflex is a human and animal behavioral motor response to an unexpected, intense stimulus. The response includes muscle contraction and closure of the eyes (Koch, 1999).

Our experiment used a sudden loud acoustic stimulus to elicit an acoustic startle response; however, tactile, visual, and olfactory stimuli can elicit a startle response (Koch, 1999). We quantified the startle reflex in mice as the full-body (jerk) of the limbs, but other investigators have used a variety of measures, such as eye-blinks in humans and escape movement in mollusks (Flaten, 2002; Mongeluzi, Hoppe, & Frost, 1998).

The acoustic startle reflex involves an ascending and descending neural pathway that begins in the auditory nerve and then travels in the following order: the ventral cochlear nucleus, the nuclei of the lateral lemniscus, the nucleus

reticularis pontis caudlis (PnC), spinal interneurons, spinal motor neurons, and then finally the flexor muscles of the face, neck, and body (Hoffman & Ison, 1980; Fitch, Threlkeld, McClure, & Peiffer, 2008).

1.2. Pre-pulse inhibition

A less-intense pre-pulse stimulus can attenuate the startle response. A pre-pulse is a stimulus that does not elicit a startle. If a subject perceives a pre-pulse presented approximately 30-500 ms before the startle-eliciting stimulus, the subject's startle reflex is attenuated (a reduced behavioral motor response). This is known as pre-pulse inhibition, or PPI. A pre-pulse can be an auditory, somatosensory, or visual stimulus (Koch, 1999). Many diverse animals show PPI, including as a few examples: humans, mice, rats, pigeons, and sea-slugs (Hoffman & Ison, 1980; Mongeluzi, Hoppe, & Frost, 1998).

PPI of the acoustic startle reflex by a variety of sensory modalities (auditory, visual, or somatosensory) involves the superior colliculus and the pedunculopontine tegmental nucleus. Auditory, visual, and somatosensory afferents project to the superior colliculus and then to the pedunculopontine tegmental nucleus. The pedunculopontine tegmental nucleus inhibits the PnC; thus, inhibiting the acoustic startle reflex. Auditory pre-pulse information projects to the superior colliculus through the auditory pathway via the inferior colliculus (Fitch, Threlkeld, McClure, & Peiffer, 2008; Koch, 1999). The inferior colliculus, particularly the lateral cortex and the dorsal cortex of the IC, are the structures of the acoustic startle-response pathway where the pre-pulse is thought to have its

inhibitory effect on the startle-response (Liuzzo, Gray, Wallace, & Gabriele, 2014; Parham & Willott, 1990).

Somatosensory inputs also project to the lateral cortex of the inferior colliculus (LCIC) (Aitkin, Dickhaus, Schult, & Zimmermann, 1978). In multiple mammalian animal models, it has been shown that somatosensory inputs from the spinal cord, dorsal column nuclei, spinal trigeminal nuclei, and the somatosensory cortex project to the LCIC. Rather than tonotopic organization, the neural auditory and somatosensory inputs to the LCIC have discrete organization. It is likely that the LCIC is the area of integration of auditory and somatosensory information (Gruters & Groh, 2012; Aitkin, Dickhaus, Schult, & Zimmermann, 1978; Cramer & Gabriele, 2014; Lesicko, Hristova, Maigler, & Llano, 2016; Loftus, Malmierca, Bishop, & Oliver, 2008). Section 1.5. further describes multisensory representation at the LCIC.

1.3. Somatosensory stimuli-elicited pre-pulse inhibition

Reports of somatosensory PPI exist for other species. For example, Mongeluzi, Hoppe, and Frost (1998) found that the marine mollusk, *Tritonia diomedea*, exhibited pre-pulse inhibition to the mollusk's escape-swim response to a tail shock when a 100 ms vibration was used as the pre-pulse. Pre-pulse inhibition of the acoustic startle response has been found in rats using somatosensory stimulation in the form of electric shock (Pinckney, 1976). There has been research conducted using somatosensory stimuli as the startle-eliciting response in mice. Brody, Dulawa, Conquet, and Geyer (2004) used an airpuff as

the startle-eliciting response in mice with an auditory or visual pre-pulse. To our knowledge, the present dissertation is the first to report vibratory PPI in mice.

1.4. Multimodal pre-pulse inhibition

There is very little literature available on multimodal pre-pulse inhibition. Brody, Dulawa, Conquet, and Geyer (2004) considered “multimodal PPI” testing in mice as presenting an auditory, visual (light), or somatosensory (puff of air) pre-pulse stimuli before an auditory, visual, or somatosensory startle-eliciting stimulus. For our current dissertation study on auditory and somatosensory PPI, we use the term “multimodal PPI” to describe a simultaneous presentation of a somatosensory (vibration) and auditory pre-pulse stimuli before an auditory startle-eliciting stimulus. Once again, to our knowledge, this dissertation is the first to record multimodal PPI in mice.

1.5. Multisensory representation at the LCIC

Aitkin, Dickhaus, Schult, and Zimmermann (1978) showed multimodal (auditory and somatosensory) inputs to the LCIC in cats. Auditory stimuli, tactile stimuli to the body, and electrical stimuli to the dorsal columns and tibial nerves were utilized to determine the activation within the LCIC. While some units of the LCIC were activated or inhibited by only one type of stimuli (auditory or somatosensory), other units were bimodally activated or inhibited. The study concluded that the LCIC accepts auditory and somatosensory input.

Jain and Shore (2006) explored the interaction between auditory and somatosensory inputs into the LCIC using guinea pigs. Electrical stimulation of

the spinal trigeminal nucleus produced no change in spontaneous activity of neurons in the lateral cortex of the inferior colliculus; when the electrical stimulation was combined with an auditory stimulus, however, there was significant changes in firing rates compared to auditory stimuli only. This shows that the projections of the trigeminal nucleus and those of the auditory system interact at the level of the LCIC.

Dehmel, Cui, and Shore (2008) displayed that somatosensory neurons innervate structures of the auditory pathway—the cochlear nucleus (CN) and LCIC. When somatosensory afferents are stimulated, both inhibition and excitation occur in second-order auditory neurons. Somatosensory influence to the CN is heightened after auditory input loss. Furthermore, animals that have been deafened have been shown to have increased spontaneous firing of CN nerves innervated by the somatosensory system. It has been speculated that these changes are a mechanism of tinnitus; therefore, somatosensory-based tinnitus treatments have been developed, but require further investigation. Some of these treatments include acupuncture, transcutaneous scalp/auricle or tempomandibular joint stimulation, and craniosacral or trigger point therapy (Dehmel, Cui, & Shore, 2008; Shore, et al., 2007).

Recent research has focused on the mechanisms and development of these multimodal projection pathways within the LCIC. Like the central nucleus of the inferior colliculus (CNIC), layer 3 of the LCIC is limited to auditory input and is tonotopically organized. It is layer 2 of the LCIC that receives multimodal input and has discrete organization with modular fields, where somatosensory inputs

project, and extramodular fields, where auditory inputs project (Cramer & Gabriele, 2014; Dillingham, Gay, Roxana, & Gabriele, 2017; Wallace, Harris, Brubaker, Klotz, & Gabriele, 2016). These modular and extramodular fields may have interconnections; thus, multisensory interactions within these structures may be possible (Lesicko & Llano, 2016). The separation of modular and extramodular fields, referred to as the "patch-matrix-like organization", of the LCIC is present in the developing mouse LCIC before onset of hearing (Dillingham, Gay, Roxana, & Gabriele, 2017; Wallace, Harris, Brubaker, Klotz, & Gabriele, 2016). The expression of Eph-ephrin receptors and ligands, which are signaling proteins that guide axonal patterning, correlates with the development of these discrete patterns. EphA4 and ephrin-B2 expression occurs within the modular patches and ephrin-B3 expression occurs within the extramodular patches during time of development (Cramer & Gabriele, 2014; Dillingham, Gay, Roxana, & Gabriele, 2017; Wallace, Harris, Brubaker, Klotz, & Gabriele, 2016). This highly correlative evidence suggests the "patch-matrix-like organization" of the LCIC and the organized-by-mode inputs to these patches are shaped by Eph-ephrin signaling. Additionally, Eph-ephrin expression is reduced as experience ensues; that is, as evoked activity begins. This leads researchers to speculate that as the system matures, Eph-ephrin signaling no longer regulates these patterns, but instead patterns are shaped by activity-dependent mechanism for each modality. There remains a need to further understand the multisensory physiology of the LCIC, how LCIC physiology relates to the modular

and extramodular organization, and the development and plasticity of LCIC organization and function (Dillingham, Gay, Roxana, & Gabriele, 2017).

1.6. Experimental purpose

Research continues to expand on the development and maturation of multimodal LCIC organizational patterns; the behavioral (functional) implications of these neural interactions, however, are unknown. Because the LCIC receives both auditory and somatosensory afferents and is involved in PPI, PPI testing (auditory, somatosensory, and combination auditory-somatosensory) was used in this study to explore the normal multi-modal psychophysical responses that might be mediated by these pathways. Eventually our goal is to explore how mutations, known to affect the development of LCIC circuits, might affect behavior. This initial report demonstrates that auditory and somatosensory stimuli, as well as their combinations, can be investigated using PPI in mice.

II. Extended Discussion

2.1. General findings

It is well established that PPI can be recorded using an auditory pre-pulse in mice (Hoffman & Ison, 1980; Koch, 1999; Liuzzo, Gray, Wallace, & Gabriele, 2014; Parisi & Ison, 1981; Fitch, Threlkeld, McClure, & Peiffer, 2008). The results from this dissertation display that a statistically significant behavioral response, in the form of PPI, can be reliably recorded using somatosensory stimuli and simultaneous auditory and somatosensory stimuli in mice. Furthermore, an age trend is present for the somatosensory PPI.

2.2. Possible origins of age trend

While mice at 108 days and older responded to the somatosensory pre-pulse, i.e. had a significant PPI, the young mice at 67 days and younger did not. This finding suggests the possibility of a heightened somatosensory response with increased age, or potentially with decline in hearing.

It is possible that the ability of mice to sense vibrations enhances with age. Mammalian tactile sensation may take weeks or months to mature after birth. In cats, tactile receptors and sensory fiber myelination likely do not reach maturity until one to two months of age, and central pathways may not mature until two to three months of age. At vibratory frequencies above 100 Hz, neonatal response thresholds are five to ten times that of adult cats (Rowe, 1982). No information was found on development of somatosensory afferents in mice.

Somatosensory PPI may not develop as quickly as auditory PPI. Parisi and Ison (1981) showed that visual PPI develops later than auditory PPI in rats. In their study, auditory PPI was present at days 13-15, but visual PPI was not present until days 21-23. Future research on the anatomical development of these multimodal afferent projections may therefore aid in our understanding of the current behavioral findings.

As for a decline in hearing, the broadband auditory stimuli presented, including frequencies up to 50 kHz for the startle stimulus and the auditory pre-pulse, is comprised of frequencies that present with hearing loss in the age ranges of the middle- and old-aged groups. C57 mice have progressive age-

related hearing loss that starts quite young, at approximately two months. This loss begins at the high frequencies, 30 kHz and above, making the high-frequency loss the most severe, and then includes middle and low frequencies as the animal ages (Li & Borg, 1991). It is also possible that hearing loss is present in the young mice group, but is far worse in the two older groups. Li and Borg (1991) used ABR testing to assess hearing in C57BL/6J mice at 2, 4, 6.3, 8, 10, 12.5, 16, 20, and 31.5 kHz. This study found that at 1 to 2 months mice have 10-23 dB loss at 12.5-31.5 kHz, the high frequency loss continues to worsen and the middle and low frequency loss progresses between 2 to 6 months and again between 7 to 9 months. At 9 to 10 months, mice have practically no hearing above 16 kHz. Ison, Allen, and O'Neill (2007) performed ABR threshold testing on C57BL/6J mice at age 7 weeks and then longitudinally every two weeks from 10 to 53 weeks. They found that at 7 weeks of age, thresholds for C57BL/6J mice are on average 50.8 dB SPL at 3 kHz, 25.4 dB SPL at 6 kHz, 7.9 dB SPL at 12 kHz, 17.5 dB SPL at 24 kHz, 22.7 at 32 kHz, and 25,4 at 48 kHz. After 10 weeks of age, the ABR thresholds begin to increase. The lower frequencies, 3 and 6 kHz, have thresholds that increase at a rate of 0.7 dB/week. Thresholds of higher frequencies, 12, 24, and 32 kHz, increase at this rate at first, but eventually increase to rates of 3-5 dB/week at week 37, 28, and 17, respectively. Thresholds at 48 kHz increase steadily at a rate of 2.3 dB/week. This raises the question concerning if a decline in hearing could possibly result in stronger sensitivity to vibrations. Perhaps neuroplasticity results in a reorganization within the LCIC when auditory input is lost, resulting in a greater representation of

somatosensory information; thus, producing a heightened response to somatosensory stimulation. Evidence for a possible increased representation of the somatosensory system with auditory deprivation, by way of increased trigeminal projections to the cochlear nucleus, was provided by Shore, et al. (2007), who found that guinea pigs with noise-damaged auditory systems had reduced (better) thresholds, decreased latencies, and enhanced response amplitudes to trigeminal stimulation, i.e. stimulation of the somatosensory pathway, and increased numbers of cells with and enhanced degrees of bimodal integration in the dorsal cochlear nucleus.

The following figure, Figure 4, is the spectrum of the SES, run through the dB ML program (Appendix 1). The figure shows the extent to which each frequency in the SES is above predicted threshold for 3 week-, 3 month-, and 9 month-old C57 mice. The model predicts a small decrease between our young and middle-aged groups. Our oldest mice were 6

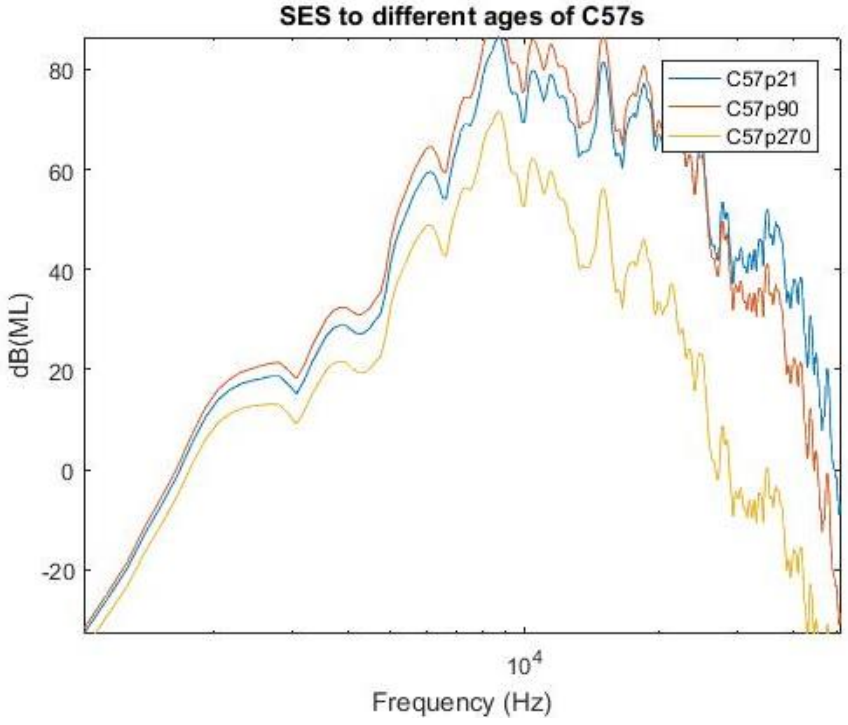

Figure 4. Predicted dB above threshold of the SES for 1, 3 and 9 month-old C57 mice.

months old and would be an unknown amount (maybe half-way) between the predictions of the 3 month-old and 9 month-old hearing losses.

An additional theory for the somatosensory age trend involves a pre-pulse inhibition maturation with age. Dean, Sheets, Crofton, and Reiter (1990) showed that with increasing age, the magnitude of pre-pulse inhibition caused by an auditory gap-detection pre-pulse also increases. For example, using a 16 ms gap pre-pulse to test mice between postnatal days 14-16 and day 65, inhibition was shown to increase from 4% to 18% with increasing age. In experienced mice that were tested for the second time and third time, inhibition increased with increasing age from 4% to 28% and 4% to 42%, respectively. At postnatal day 65, the magnitude of inhibition with the 16 ms gap pre-pulse was asymptotic with increasing age. As one can see, a learning-effect was also present in the older mice. It may be possible that some or all of the young mice, at 30 – 67 days old, had immature PPI pathways during testing. Perhaps further research on age trends and somatosensory PPI is warranted.

2.3. Arguments against the above concepts on age trends

Some of our data oppose the possibility of a significant hearing loss over the ages we studied. Figure 3, within the Results section of Part I (responses to the 25 ms stimuli over age), shows the greatest response to the auditory pre-pulse in the oldest group, the opposite of what would be predicted from an age-related hearing loss. Perhaps any decline in hearing would be more evident in responsiveness to the shortest stimulus. Figure 5, below, is similar to Figure 3 only for the shortest (4ms) pre-pulses. There is no decrease in responsiveness

at the oldest ages, so age-related hearing loss, though definitely a possibility does not seem to be a compelling factor in age-related trends in these data.

An argument also exists against the concept that a decline in hearing results in stronger vibrotactile sensitivity. It has been found that humans have a decrease in vibrotactile sensation as they age (Verrillo, 1980). An increased representation of somatosensory afferents would not occur if somatosensory input and auditory inputs were both reduced with age. Yet, the possibility of an increase in vibrotactile threshold and the timeline for such is unknown in mice. Once again, these findings warrant further research on the anatomical progression of the multimodal afferent system.

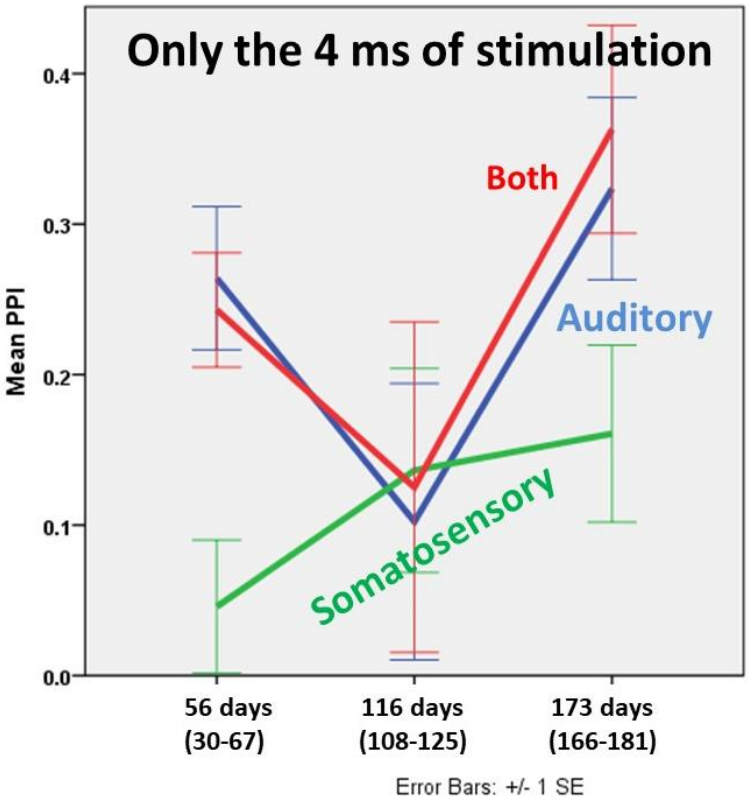

Figure 5. PPI trends with age (young, middle, and old) for the 4 ms auditory (blue), somatosensory (green), and both simultaneously (red) pre-pulses only.

2.4. Somatosensory thresholds and their influence on PPI

Concerning the somatosensory PPI of the middle- and old-aged mice, the mice were responsive only to the 500 Hz and 360 Hz vibrations, but particularly the 360 Hz vibration. In humans, thresholds of vibration are different across

frequencies (Verrillo, 1980). The varying thresholds by frequency are seen in Figure 6, redrawn from Verrillo (1980). As one can see, thresholds are greatest in the lowest frequencies, slope down and become the least in the mid frequencies, and rise to be greater (but not as great as the lowest frequencies) in the highest frequencies. It is likely that the thresholds of vibration for each frequency are different for mice as well. This may explain why only 500 Hz and 360 Hz elicited PPI and the other frequencies did not. The intensity of all vibrations was kept constant; therefore, it is possible that the constant intensity level was below the vibrotactile thresholds for 460 and 220 Hz but was above the threshold levels at 500 and 360 Hz (Verrillo, 1980). Therefore, with recent research on dB ML (hearing thresholds of mice), perhaps we could also expand into research on dB ML in terms of vibrotactile thresholds (see Appendix 1). The responsiveness of middle- and old-aged mice to 360 Hz vibrations, and the low or lack of response to the higher and lower frequencies, suggests a U-shaped curve for vibratory thresholds as a function of frequency.

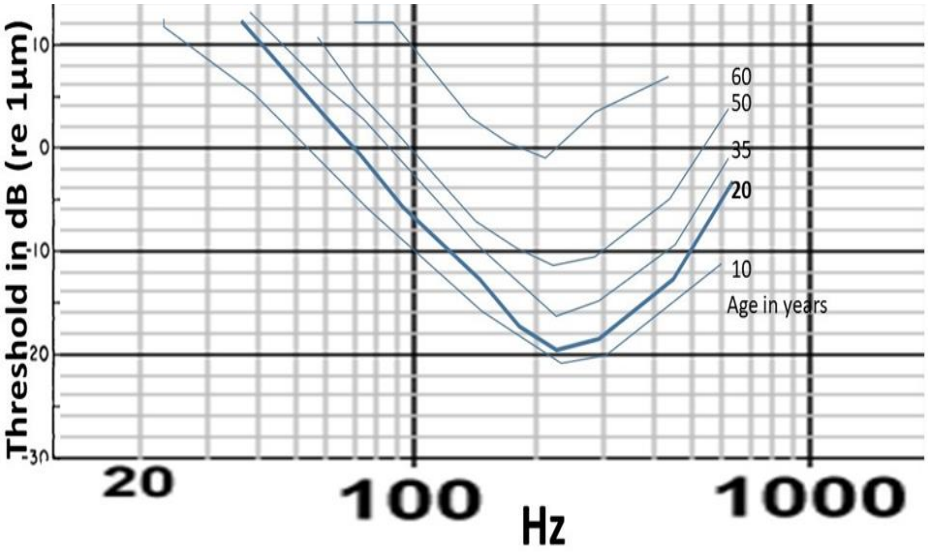

Figure 6. The varying thresholds of vibration by frequency in humans, redrawn from Verrillo (1980).

Figure 7 below is a crude attempt to put the data from Figures 2 and 6 on the same graph. The somatosensory responses of the mice in Figure 2 above were 'flipped' in both dimensions. The responses of the mice are graphed below as a function of the frequency of the vibration, not the duration (frequencies of the vibrations decreased as durations increased as seen in Table 1 of the Methods section in Part I). Further, higher PPI would suggest a lower threshold, so a log and inverse transformation, $\log_{10}$ (1-PPI), was used to invert the Y axis, and then this value was multiplied by 150 (arbitrary rescaling to get values for both species into roughly the same range). These mouse data are plotted with data from the 20 year-olds from Verrillo (1980). The x-axis is frequency (Hz); the Y-axis is threshold in dB for the humans, and the transformed and scaled PPI values for the mice. This preliminary attempt to relate somatosensory thresholds in both species is

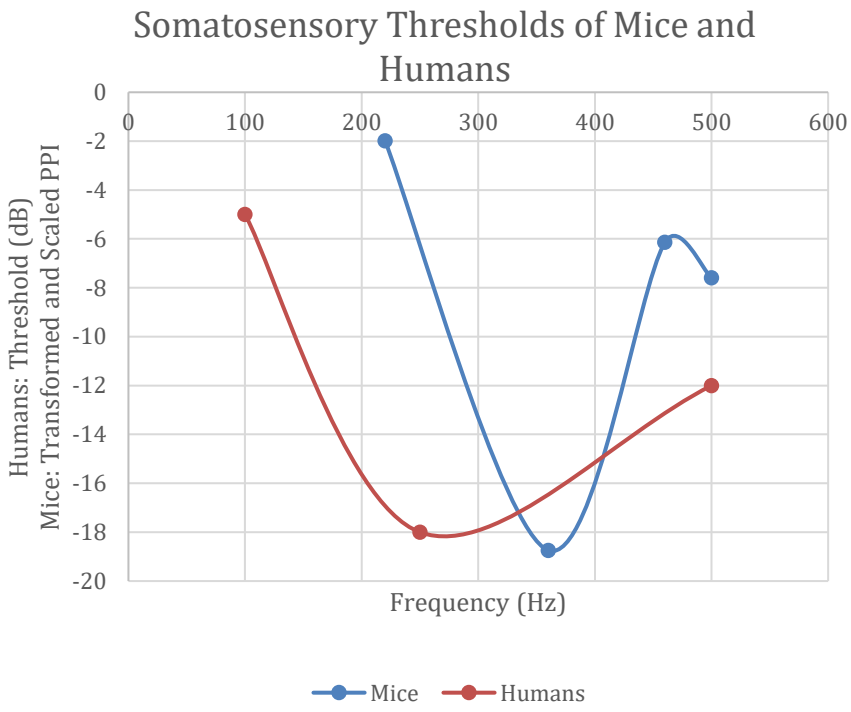

Figure 7. Mouse PPI as a function of frequency of vibration (results of present dissertation study) compared to vibratory thresholds of 20-year-old humans (taken from Verrillo (1980) study).

consistent with a ‘best’ middle frequency and decreasing responsiveness at higher and lower frequencies of vibrations.

In addition to expanding current research to dB ML in terms of vibrotactile thresholds, vibrotactile thresholds with age in mice could also be explored. As mentioned above, humans have a decrease in vibrotactile sensation as they age. Verrillo (1980) found the vibrotactile thresholds for 25, 40, 64, 80, 100, 160, 200, 250, 320, 500, and 700 Hz in humans of five age groups with mean ages of 10, 20, 35, 50, and 65 years. Verrillo found that vibrotactile thresholds at the lowest frequency tested, 25 Hz, were the same for all age groups. Additionally, the 40 Hz threshold barely changed with age. Mid frequencies, such as 80 Hz, had a consistent increase (3 dB every decade of life). The high frequencies, such as 160 and 250 Hz, had a threshold increase that increased by a greater amount at every decade of life. When thresholds did increase with age, they did not increase past the threshold of 25 Hz.

Gescheider, Bolanowski, Hall, Hoffman, & Verrillo (1994) similarly showed that vibratory thresholds increase with age in humans. Additionally, their study showed that females at 20 years old and older had better vibratory thresholds than males of the same age. While they also found that higher frequencies are affected more than low frequencies, in contrast to Verrillo (1980), their results displayed an increase in the thresholds of the lowest frequencies tested (1 and 10 Hz) with age. Furthermore, they found that threshold increase was constant with increasing age, and approximately the same for males and females, until

age 65 years. After age 65 the increase occurred at a more advanced rate, even more so for males.

For our research in mice, determining vibrotactile thresholds across frequencies, the increase in thresholds with age, and the timeline for such an increase would aid in future testing of the behavior of the multimodal afferent system. Exploring gender differences may also be helpful. This would ensure that future testing was performed with somatosensory stimuli that were suprathreshold. Also, testing across frequencies could be more efficient and productive, because rather than testing for somatosensory PPI at a constant intensity level across frequencies, a constant intensity level above threshold for each frequency could be used.

2.5. Final conclusions

The simultaneous 9 cycles of the 360 Hz vibration and the 25 ms long broadband sound resulted in an additive-like effect, in that the multimodal PPI was very close to, but not precisely, the sum of the PPI to each modality alone. This behavioral finding supports the theory that auditory and somatosensory afferent pathways can converge to affect responsiveness to startling stimuli.

The goal of this dissertation was to use multimodal pre-pulse inhibition to provide the behavioral component to current research exploring the development and maturation of multimodal LCIC organizational patterns. These results provide some understanding of multimodal sensory interaction in mice with normal auditory and somatosensory pathways through the LCIC. Furthermore,

this research has provided a behavioral paradigm to assess functional consequences of somatosensory/auditory interactions in mice. The overall aim of the NIH grant that supported this work is to explore how Eph-ephrin mutations might compromise multimodal neural circuits and neural processing. We expect PPI in mutants to differ from PPI findings in normal mice. It has been shown that homozygous, EphA4$^{lacZ/lacZ}$, and knockout ephrin-B3, ephrin-B3 $^{+/-,\ -/-}$, mice have reduced auditory PPI compared to control and heterozygous, EphA4$^{lacZ/+}$, mice. EphA4 and ephrin-B3 are essential in development of auditory behavioral circuits; thus, the homozygous and knockout mutations resulted in reduced auditory PPI (Liuzzo, Gray, Wallace, & Gabriele, 2014). As stated earlier, during development EphA4 and ephrin-B2 expression occurs within the modular (somatosensory) patches, and ephrin-B3 expression occurs within the extramodular (auditory) patches during early postnatal development (Cramer & Gabriele, 2014; Dillingham, Gay, Roxana, & Gabriele, 2017; Wallace, Harris, Brubaker, Klotz, & Gabriele, 2016). If these Eph-ephrins provide guidance signals that influnce the development of discrete somatosensory and auditory LCIC patterns, it is expected that mutant mice with compromised Eph-ephrin interactions will have corresponding reduced auditory, somatosensory, and/or multimodal PPI.

Appendices

Appendix 1: The dB ML program used in deciding which vibratory stimuli produced no audible sounds to the mice

```
%dBMLall %documentation and functions to estimate 'Mouse Level' of
sounds. %dBML returns decibels 'Mouse Level', dB above estimated murine
thresholds (similar to dB HL for humans). %Acknowledgements: this work
was supported by NIH R15 grants DC012421  to Dr. Mark Gabriele and
DC015353 to M.G. & L.G.; %Dr. Christopher Clinard provided helpful
comments as well as parts of  the code for the real-time spectral
analysis.

%calling convention is [out1, out2]=dBML('strain', [optional pairs])
%The first input argument 'strain' is required
  % can be 'C57', 'CBA', 'C57p21', 'C57p90', 'C57p270', 'Human' or
 'dBSPL'
     %(the last two options obviously not strains of mice)      %C57 is
the same as C57p21. p means post-natal day (=age). %A single output can
be dBML (dB>threshold), TF (can mouse hear  input?), or threshold
(spectrum)    %the single output argument depends on the input arguments
as  described below     %dB=dBML('strain','Hz',freq) %returns the
threshold for the  specified strain and frequency.
%Q=dBML('strain','Hz',x,'dB',y) %returns 1 if the strain can hear  the
frequency x at level y.    %Q=dBML('strain','File','csvFile') %returns 1
if the sounds  specified in a spectrum (Hz,dB pairs) input as a csv file
can be  heard     %Out2xN=dBML('strain') %returns 2xN matrix: the
expected audiogram  of the strain(doesn't work for 'dBSPL') %A second
output argument can include the spectrum: many paired values  of Hz and
dB in a two-by-N matrix  %[Q,OutN2]=dBML('strain', ...) %Q=1 if
max(Out2xN(:,2)>0
%Optional input arguments can include the following in the typical
 Matlab 'parameter', value pairs
    %'Hz', number
        %the number after 'Hz' is a frequency
    %'dB', number         %the number after 'dB' is a level, used with
Hz, such as can a  mouse hear a frequency at that level?     %'file',
'string'         % after 'file' is name of a .csv file with vertical
pairs of  frequencies in Hz and intensities in dB (SPL)     %'RealTime',
'string'
        %after 'RealTime' can be 'Default','B&KHF,'B&KHalfInch','ER7-
C' or 'NTI'         %an input voltage is immediately
digitized, and dBML  determines whether that signal can be
heard by a mouse         %various other input-argument pairs
can follow the
 'RealTime','something' pair             %'ms', number -- duration of
the recording to be analyzed  in ms (defaults to 500)
            %'Fs', number -- sampling frequency of the recording in Hz
%'calV', number -- V(RMS) of a calibration tone at level  specified by
caldB           %'caldB', number -- dB that produces calV in a recording
 (defaults to 94)
```

```
% Examples
%
% Example 1: Graph the expected thresholds of the different strains
(these calculations described in a paper under review) clear; clc;
strain1='CBA'; strain2='C57'; strain3='Human'; %or get predicted age-
related decline of C57s
%strain1='C57p21'; strain2='C57p90'; strain3='C57p270';
Out1=dBML(strain1);
Out2=dBML(strain2); Out3=dBML(strain3); semilogx(Out1(1,:),Out1(2,:))
hold on semilogx(Out2(1,:),Out2(2,:)) semilogx(Out3(1,:),Out3(2,:))
legend(strain1,strain2,strain3) xlabel('Frequency (Hz)');
ylabel('dB'); title('Example 1: predicted  audiograms of various
strains'); snapnow disp('Example 1 outputs are in Figure 1')

%Examples 2 and 3: find the threshold at a particular frequency clear;
dBOut=dBML('C57','Hz',1000); %returns expected threshold of 70 dB at
1kHz for C57 mice disp(['Example 2: expected 1kHz thresholds of C57
mice is '  num2str(dBOut)]);

%can a mouse hear a specified tone?
Q=dBML('CBA','Hz',8000,'dB',10); %returns 0 because the threshold at
8k is 20 dB disp(['Example 3: CBA mice can ' char('Not' *~Q) ' hear
8kHz at 10  dB'])

%Example 4: is a previously recorded spectrum (saved as csv) audible?
clear; strain='CBA'; filnam='csvInput.txt';
Q=dBML(strain,'File',filnam); %returns 0 because all Hz/dB pairs in
the file are below threshold disp(['Example 4a: ' strain ' mice can '
char('Not' *~Q) ' hear the  sounds in ' filnam])

[Q,Out2xN]=dBML(strain,'File',filnam);
figure semilogx(Out2xN(1,:),Out2xN(2,:))
xlim([min(Out2xN(1,:)) max(Out2xN(1,:))])
ylim([min(Out2xN(2,:)) max(Out2xN(2,:))])
xlabel('Frequency (Hz)'); ylabel('dB(ML)');
title(['Example 4b: sounds  in ' filnam '
in dB re ' char(strain) ' threshold']);
disp('Example 4b is in Figure 2')

%Examples 5-9 require an input voltage from a calibrated microphone
%call dBML with parameter 'RealTime' set to
 'Default','B&KHF,'B&KHalfInch','ER7-C' or 'NTI'     %default was tested
with a RealTek microphone array on 100% gain  and 10% boost. Dell
Latitude E5550     %94 dB cal tone from B&K4230 gave .138Vrms when half-
inch coupler  placed over left of two small top holes disp(' ')
disp('The following require voltages to be recorded by this program;
code will likely need revision for your setup (maybe drastic)') clear;
figure; [~,Out2xN]=dBML('SPL','RealTime','Default','Fs',8000);
[peak,ind]=max(Out2xN(2,:)); disp(['Example 5; peak of ' num2str(peak) '
dB at '  num2str(Out2xN(1,ind)) ' Hz']) disp('raw data from default mic
are in Figure 3') %else you need write function data=getData(Fs,npoints)
to return  npoints data at Fs sampling rate %the function with this
submission used TuckerDavis RZ6 input B. %all these calibrations were
```

```
checked with a B&K4230 94 dB, 1kHz  calibrated sound source %'B&KHF'
would be a good mic to measure the high frequencies mice can  hear;
%with a B&K4939microphone, Listen Inc. SoundConnect Amp with A1 A2  at
20 dB, A3 at 0 dB.    % 94 dB cal tone gives 357mV rms, so 'calV',367.E-
3
%'B&KHalfInch would be good for humans and frequencies up to 20kHz
% B&K 4176 mic with B&K 2235 SLM on 40-110 dB; 94 dB cal tone  gives
163.875 mVRMS %'ER7-C' is a useful small probe-tube microphone
    % 'calV',5.E-2, for Etymotic ER7-C on 0 dB %to get calV you could
measure peak-to-peak voltage of sine wave on an  oscilloscope and divide
by 2*sqrt(2) or 2.8284 %first check that you get the correct dB (SPL)
from a known sound as  in the following example clear; try
[~,Out2xN]=dBML('SPL','RealTime','B&KHalfInch');
[maxdB,index]=max(Out2xN(2,:)); disp(['Example 6: cal tone gave '
num2str(maxdB) ' dB at '  num2str(Out2xN(1,index)) ' Hz']) figure
semilogx(Out2xN(1,:),Out2xN(2,:)) xlim([min(Out2xN(1,:))
max(Out2xN(1,:))]) ylim([min(Out2xN(2,:)) max(Out2xN(2,:))])
xlabel('Frequency (Hz)'); ylabel('dB(ML)'); title(['dB SPL']);

%can a mouse hear an input?
Q=dBML('C57','RealTime','B&KHF');

%note that if only 1 output arg, dBML plots input in time and
frequency domains disp(['mouse CAN ' char('NOT'*~Q) ' hear the
input'])

%get more information about an input, like a real-ear measurement
clear; strain='Human'; [Q,Out2xN]=dBML(strain,'RealTime','ER7-C');
if Q     [maxdB,index]=max(Out2xN(2,:));     disp([strain 's CAN
hear this sound, with a max of '  num2str(maxdB) ' dB at '
num2str(Out2xN(1,index)) ' Hz']) else     disp([strain 's can NOT
hear this sound']) end figure plot(Out2xN(1,:),Out2xN(2,:))
ylim([min(Out2xN(2,:)) max(Out2xN(2,:))]) xlabel('Frequency (Hz)');
ylabel('dB(ML)'); title(['dB above '  char(strain) ' threshold']);
catch     disp('Something went wrong with the real-time recording')
end

function data=getData(Fs,npoints) %this is VERY dependent on individual
set up %this particular version for TDT RZ6 fake=0; %set to 1 just for
off-line debugging pub=1; %set to 1 to get Matlab >publish
('dbMLall.m','pdf') to run if ~fake     try         RZ6 =
actxcontrol('RPco.x', [10, 5, 36, 26]);         if RZ6.ConnectRZ6('GB',
1)             disp('connected to external ADC device');         else
disp('failed to connect to your ZZ6');             return         end
catch         disp('unable to connect to external ADC device');
disp('try calling dBML(''Human'',''Realteime'',''Default'')')
return     end     rpvdsFile='wRZ6AudioInput.rcx';     if Fs ~=
24414.062500         disp(['change sampling rate in ' rpvdsFile ' to '
num2str(Fs) ...             ' or change Fs in the Matlab calling program
to  24414.0625'])         return     end     if npoints > 10000
disp(['change nHi, BlkSze, Size in ' rpvdsFile ' to be at  least '
num2str(npoints)])
```

```
        return     end     RZ6.Halt; % Stops any processing
chains running on RP2
    RZ6.ClearCOF; % Clears all the buffers and circuits on that RP2
    RZ6.LoadCOF(rpvdsFile);     RZ6.Run();
status=double(RZ6.GetStatus); % Gets the status     if
bitget(status,1)==0; % Checks for connection         disp('Error
connecting to RZ6'); return;     elseif bitget(status,2)==0; % Checks
for errors in loading circuit         disp(['Error loading '
rpvdsFile]); return;     elseif bitget(status,3)==0 % Checks for errors
in running circuit         disp(['Error running ' rpvdsFile ' on RZ6']);
return;     else         disp(['RZ6 successfully running ' rpvdsFile]);
end     pause('on')     if ~pub; input('press any key to measure
sound'); end     if ~RZ6.SoftTrg(1); error ('SoftTrg error!'); end
pause(ceil(npoints/Fs));     tempA=RZ6.ReadTagV('AudioAin', 0, npoints);
%read the data %read  the data     AdcA=tempA(2:npoints);
DCoffset=mean(AdcA);     data=AdcA-DCoffset;     disp(['Vmax = '
num2str(max(abs(AdcA))) ' RMS = '  num2str(rms(data)) '; DC = '
num2str(DCoffset)]) else     data=rand(1,npoints); end end
```

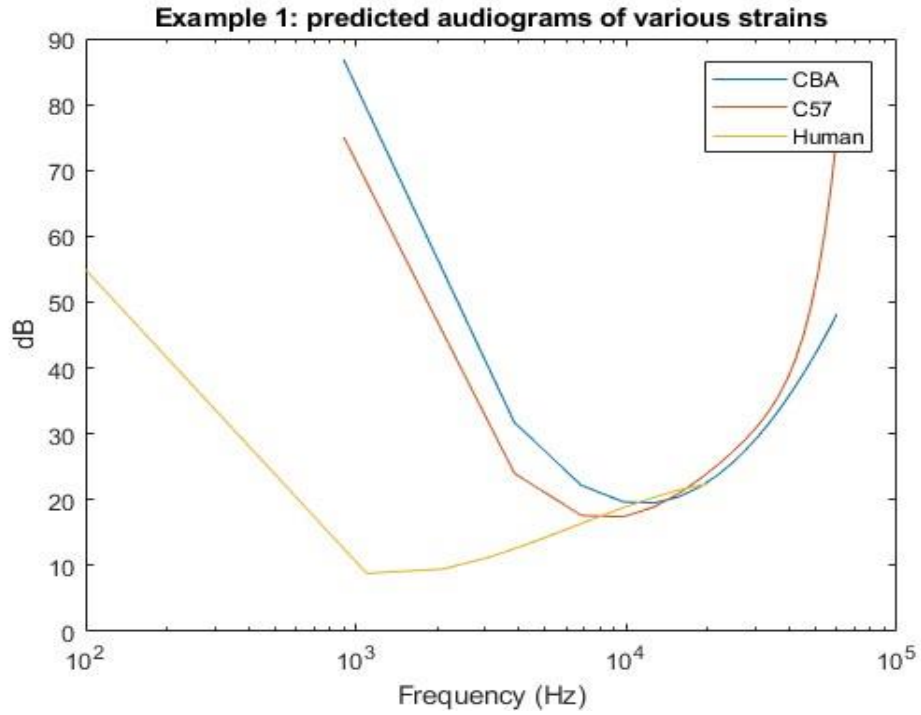

Figure 1. The expected thresholds of the different strains.

Example 1 outputs are in Figure 1
Example 2: expected 1kHz thresholds of C57 mice is 69.7519
Example 3: CBA mice can Not hear 8kHz at 10 dB
Example 4a: CBA mice can Not hear the sounds in csvInput.txt
Example 4b is in Figure 2
The following require voltages to be recorded by this program; code will likely need revision for your setup (maybe drastic) Example 5; peak of 52.662 dB at 90.045 Hz raw data from default mic are in Figure 3

```
unable to connect to external ADC device try calling
dBML('Human','Realteime','Default') looks like you need to write or
rewrite function getData to collect  real-time data on your
particular system
Something went wrong with the real-time recording
```

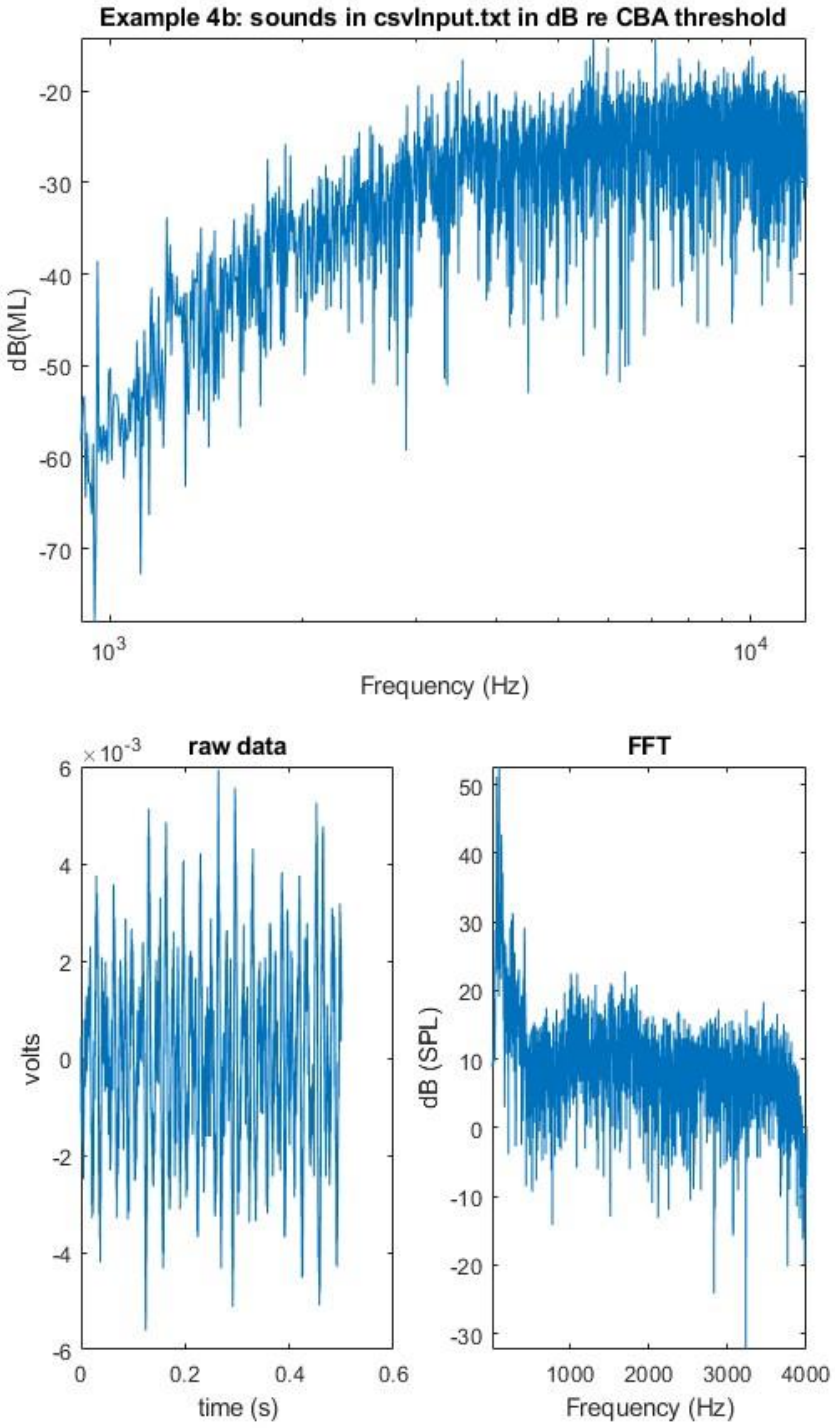

Figure 2. Example 4b outputs.

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Appendix 2: The Gray manuscript describing the data used to produce the dB ML program (Appendix 1 above)

Automated measurement of sounds relative to the hearing threshold of laboratory mice: 'dB ML'

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Keywords: mouse, audiogram

Abstract

A method was created to predict the level of sounds relative to hearing thresholds of laboratory mice. Polynomials are fit to existing data on the hearing thresholds of CBA and C57 strains of mice. Computer code (Matlab function dBML, available through the matlabcentral public file exchange) uses these polynomials to output the degree to which inputs about sounds (either specified levels of various frequencies or 'real-time' voltages digitized from a calibrated microphone) are above the hearing level of these strains of mice.

Introduction

Mice have long been a popular animal model for hearing research (Willott, 2001). As automation in laboratories increases it is sometimes desirable to predict (or check) the level of sounds relative to the hearing threshold of laboratory mice. This would be similar to the dB HL scale (American National Standards Institute, 1996), where "clinicians measure sound intensity in dB

HL (decibels Hearing Level), i.e. dB relative to the quietest sounds that a young healthy individual ought to be able to hear." (Schnupp, Nelken, & King, 2018).

The present paper presents a method for automated measurements of what we call 'decibels of Mouse Level', or dB ML, the level of a sound relative to a mouse's thresholds. Many experiments use the CBA strain of laboratory mouse (Berlin, 1963; Birch, Warfield, Ruben, & Mikaelian, 1968; Prosen, Dore, & May, 2003; Radziwon et al., 2009). The C57 strain is a common 'background' for many genetic manipulations, but this is somewhat problematic for auditory researchers because this strain loses high frequency hearing quickly. Extensive data on physiological response thresholds from both CBA and C57 mice are included in a paper on auditory brainstem evoked potentials (Zheng, Johnson, & Erway, 1999), and these data are used to adjust the fit from CBAs to be more appropriate to C57 mice of various ages.

Methods

Most available behavioral data on hearing thresholds of mice are from young adults of the CBA strain. Figure 1 shows the data used in this analysis. The curve fit app in Matlab (V2017a; Mathworks Inc, Natick, MA) fit polynomial regressions to these data. Reasonably simple polynomials with good fit were subjectively selected from among the many available, more complex options. Because of the considerable variability among published reports of CBA thresholds seen in Figure 1, a more conservative estimate was also made; 18 points along the bottom of the figure, not including what seems to be an outlier at

2000 Hz and 0 dB, were fit to a 2-component Fourier series, termed ‘min’ for minimum threshold.

Limited behavioral data on thresholds of C57 mice were found, but there is extensive data on ABR thresholds for CBA and various ages of C57 mice (21, 90 and 270 days) (Zheng et al., 1999). ABR thresholds are typically different from behavioral thresholds (higher at high frequencies and lower at low frequencies) (Davis & Ferraro, 1984; Gorga, 1999; Heffner H. E & Heffner R. S, 2003), but the relationship between strains should be the same when measured physiologically and behaviorally. These physiological data (Zhang et al., 2013) were used to correct the CBA thresholds derived above for C57 strains. Figure 2 shows these corrections. Such corrections have worked well to predict behavioral thresholds from ABR thresholds in humans (Stapells, 2000; Vander Werff, Prieve, & Georgantas, 2009) .

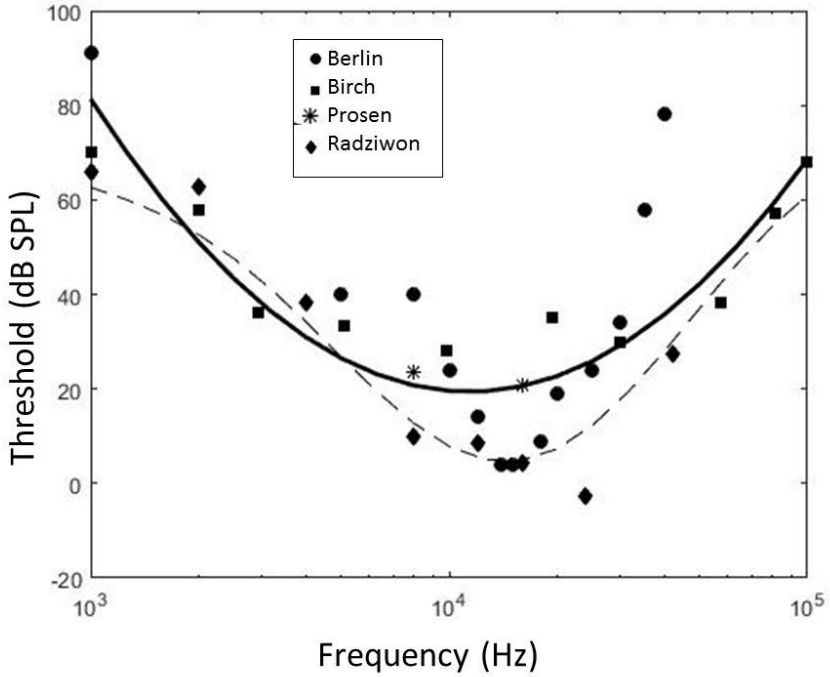

Figure 2 Data used and the CBA (solid line) and 'min' (dashed line) fits.

Results

Table 1 shows the regressions and the variance accounted for by each. Figure 1 shows the CBA regression by the solid line and the 'Min' regression in the dashed line. Predicted threshold of the C57 mice are seen in Figure 3.

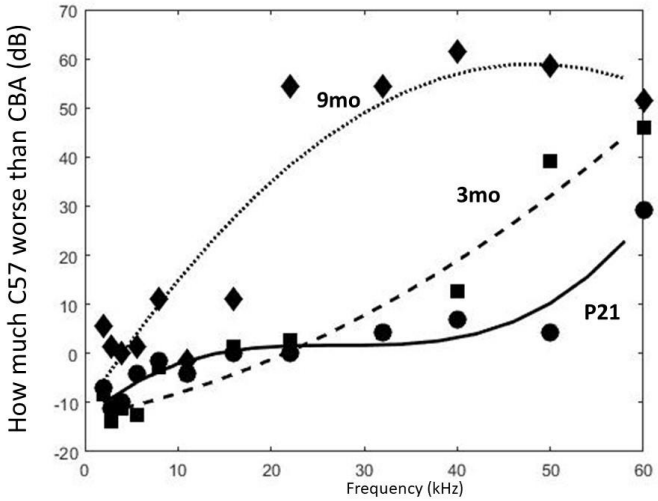

Figure 3 Differences between CBA and C57 ABR thresholds calculated from data of (Zhang et al., 2013)

Table 1. Polynomial fits to the data. logHz is base-10 logarithm of frequency in Hz

Strain	Fit	r^2
CBA	$55.38*logHz^2 - 449.4*logHz + 931.1$;	.64
C57p21	$CBA + 0.00073*kHz^3 - 0.06*kHz^2 + 1.63*kHz - 13.14$	.92
C573m	$CBA + 0.01057*kHz^2 + 0.3701*kHz - 12.88$	.91
C579m	$CBA - 0.03034*kHz^2 + 2.921*kHz - 11.32$	.87
Min	$42.8+27.9*\cos(x*w)-15.8*\sin(x*w)-5.8*\cos(2*x*w)$ $+3.3*\sin(2*x*w)$ where x=logHz and w=2.17	.93

Discussion

Good fits to existing data are obtained with relatively simple regressions (< 7 terms) for CBA and various strains of C57 mice. Figure 3 shows the predicted

thresholds of C57 mice at various ages. Added are unpublished data from a hybrid of C57 with C3HeB/Fej mice (Heffner H. E & Heffner R. S, 2003); the C3HeB strain has lower high-frequency thresholds than C57s (Zheng et al., 1999), so the estimates seem reasonable.

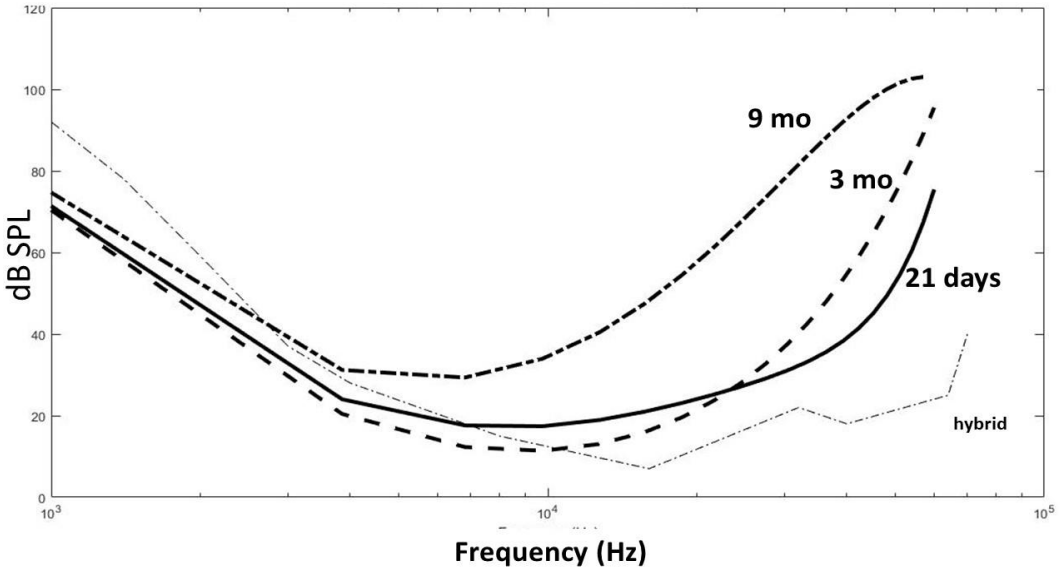

Figure 4 Predicted thresholds of C57 mice.

A Matlab function, dBML, implements these predictions. The source code and thorough documentation and source code is available as supplementary material to this paper and can be downloaded free of change at https://www.mathworks.com/matlabcentral/. Below is abbreviated documentation:

```
dBML returns decibels 'Mouse Level', dB above estimated murine
thresholds. The calling convention is [out1,out2]=dBML('strain',
[optional pairs])
The first input argument, 'strain', is required and can be can be
'C57', 'CBA', 'C57p21', 'C57p90', 'C57p270', 'Human' or 'dBSPL'.
(C57=C57p21)
```

```
Output(s) can be dB ML (dB>threshold), TF (can mouse hear input?), or
the spectrum (Hz, dB pairs of predicted threshold).
For example:
dB=dBML('strain','Hz',freq) %returns threshold for input strain and Hz
Q=dBML('strain','Hz',x,'dB',y) %returns 1 if strain can hear x Hz at y
dB SPL
Out2xN=dBML('strain') %returns 2xN matrix, expected audiogram of the
strain
Optional input arguments can include the following in the typical
Matlab 'parameter', value pairs
```

- 'Hz', number %the number after 'Hz' is a frequency
- 'dB', number %the number after 'dB' is a level, used with Hz
- 'file', 'string' % a .csv file with vertical Hz, dB pairs
- 'RealTime', 'string' % records data from a calibrated microphone
    - The sting after 'RealTime' can be 'Default', 'B&KHF, 'B&KHalfInch','ER7-C' or 'NTI'
    - various other input-argument pairs can follow RealTime' pair
        - 'ms', number %duration of recording (default = 500)
        - 'Fs', number %sampling frequency of the recording in Hz
        - 'calV', number %V(rms) of a calibration tone
        - 'caldB', number %dB SPL that produced calV (default = 94)

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Louthan provided helpful comments, and C.C. wrote parts of the code for the real-time spectral analysis.

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Appendix 3: The MM3 Matlab program that resented the multimodal stimuli and recorded the PPI

```
%MM3
%Multimodal, with 'best' stim Mar 2016
clear;
clc;
%these are the parameters to CHANGE on every run
comment='MM3 Old Guy';
filename='MMOGUTc';

debug=0; %0 for a real run
%these are parameters that would change from debugging to running
if debug
    nBlocks=1; %should be 11 to replicate Ison
    acclim=0*60; %3 min acclimation to chamber before test
    ITIs=[2 2];  %ITI low and high limits 15 to 25
    SoftStartle=1; %should be 1, except .1 for 20 dB less startle for
testing
    saveIt=0; % 1 to save data
    dBPA5=10; %40dB down should give 40 dB SPL
else
    nBlocks=11; %11 blocks of 16 = Ison = 176 total.
    acclim=4*60; %3 min acclimation + 1 min to insert the mouse
    SoftStartle=1; %should be 1, except .1 for 20 dB less startle for
testing
    saveIt=1; % 1 to save data
    ITIs=[15 25];  %ITI low and high limits 15 to 25
    dBPA5=10; %70 dB BBN from MMTone.rcx GausNoise Amp =.099
end
clock
start=rem(now,1);
%these are the parameters that should NOT change
ms2Meas=100; %duration to record accelerations after the stimulus
ms4ES=15; noSES=0; %does the mouse get startled or not
fs = 48828.125; %running at 50kHz
pnts2Meas=ceil(ms2Meas*fs/1000);
inchesAway=6; %distance from speaker to
mouseSpeedOfSoundDelay=ceil(.0737*inchesAway*fs/1000);
SpeedOfSoundDelay=floor(.0737*inchesAway*fs/1000);
ADCdelay=35+SpeedOfSoundDelay; %should be 65-30 for RP2.1 on p 51 of
RPvdsEx_Manual.pdf.
%TestBlock is SES?, #vibcycles, Tone, msISI, vibHz
if debug
    TestBlock=[ms4ES 2 20 200 100; ...  %2 cycles of 100 Hz = 20 ms
               ms4ES 5 20 200 100; ...
               ms4ES 2 20 300 100; ...
               ms4ES 5 50 100 100; ...
               ms4ES 5 50 200 100; ...
               ms4ES 5 50 300 100; ....
               ms4ES 0  0 100 100; ....
               ms4ES 0  0 200 100;
               ms4ES 0  0 300 100];
else
    TestBlock =[ms4ES  9 25 360 200; ...  % SES buzz tone Hz ISI
                ms4ES  4  9 460 200; ...  %
```

```
                ms4ES  2  4 500 200; ...
                ms4ES 10 45 220 150; ...
                ms4ES  9  0 360 200; ...  %buzz alone
                ms4ES  4  0 460 200; ...
                ms4ES  2  0 500 200; ...
                ms4ES 10  0 220 150; ...
                ms4ES  0 25 360 200; ...  %tone alone
                ms4ES  0  9 460 200; ...  %
                ms4ES  0  4 500 200; ...
                ms4ES  0 45 220 150; ...
                ms4ES  0  0   0 200;
                ms4ES  0  0   0 200
                noSES  0  0   0 200];
end
nTrials=length(TestBlock);
data=zeros(nBlocks*nTrials,7); %allocate matrix for data, assuming 10
trials per block: block trial PP startle RMS VPtP

%and now we start the two RP2s
RP1st=actxcontrol('RPco.X',[5 5 26 26]);
RP1st.ConnectRP2('GB', 1);
RP1st.Halt; % Stops any processing chains running on RP2
RP1st.ClearCOF; % Clears all the buffers and circuits on that RP2
RP1st.LoadCOF('MMPPI1.rcx');
RP1st.Run();
status=double(RP1st.GetStatus); % Gets the status
if bitget(status,1)==0; % Checks for connection
    disp('Error connecting to first RP2'); return;
elseif bitget(status,2)==0; % Checks for errors in loading circuit
    disp('Error loading circuit for first RP2'); return;
elseif bitget(status,3)==0 % Checks for errors in running circuit
    disp('Error running circuit for first RP2'); return;
else
    disp('1st RP2 is running');
end
RP2nd=actxcontrol('RPco.X',[5 5 26 26]);
RP2nd.ConnectRP2('GB', 2);
RP2nd.Halt; % Stops any processing chains running on RP2
RP2nd.ClearCOF; % Clears all the buffers and circuits on that RP2
RP2nd.LoadCOF('MMTone.rcx');
RP2nd.Run();
status=double(RP2nd.GetStatus); % Gets the status
if bitget(status,1)==0; % Checks for connection
    disp('Error connecting to second RP2'); return;
elseif bitget(status,2)==0; % Checks for errors in loading circuit
    disp('Error loading circuit for 2nd RP2'); return;
elseif bitget(status,3)==0 % Checks for errors in running circuit
    disp('Error running circuit for 2nd RP2'); return;
else
    disp('2nd RP2 is running');
end
 %now connect to the PA5, programmable attenuator.
    PA5x1=actxcontrol('PA5.x', [5 5 26 26]); % Connects to PA5 via GB
    if (PA5x1.ConnectPA5('GB', 1)==1)
        disp('PA5 is connected')
        PA5x1.Display('ForPPI', 0);
        q=PA5x1.SetAtten(dBPA5);
```

```
    else
        disp('Unable to connect to PA5');
    end

% connect to the Agilent Function Generator
% Find a VISA-GPIB object.
obj1 = instrfind('Type', 'visa-gpib', 'RsrcName',
'GPIB0::10::0::INSTR', 'Tag', '');
% Create the VISA-GPIB object if it does not exist
% otherwise use the object that was found.
if isempty(obj1)
    obj1 = visa('AGILENT', 'GPIB0::10::0::INSTR');
else
    fclose(obj1);
    obj1 = obj1(1);
end
% Connect to instrument object, obj1.
fopen(obj1);
% Communicating with instrument object, obj1.
fprintf(obj1, 'FUNC SIN');
fprintf(obj1, 'FREQ 100 HZ');
fprintf(obj1, 'VOLT 1.5 VPP');
fprintf(obj1, 'BURS:MODE TRIG');
fprintf(obj1, 'TRIG:SOUR BUS');
fprintf(obj1, 'BURS:STAT ON');
fprintf(obj1, 'OUTP:TRIG ON');

%***redo the next statement in this and GD.m!!!!!!!!!!
maxms=max(TestBlock(:,5))+ ms2Meas;
maxPoints=ceil(fs*maxms/1000)+ADCdelay+1;
% err=RP1st.SetTagVal('BlkSze',maxPoints);
% if ~err
%     disp('error setting BlkSze')
% end
raw=zeros(nBlocks*nTrials,maxPoints);
time=(1:maxPoints)/fs;

pause on;
pause(acclim);
tR=1;
nOvld=0;
maxmax=0;
for block=1:nBlocks
    %shuffle the trials
    Sort=[TestBlock rand(length(TestBlock),1)]; %4rd column is random
numbers
    Shuffled=sortrows(Sort,4); %sort by those random numbers,
effectively shuffling the trials
    for trial=1:nTrials
%         msOfBuzz=Shuffled(trial,2)*10;
%         msISI=Shuffled(trial,4)+2.4+msOfBuzz; %stim + wait
        %set up for the test
        msISI=Shuffled(trial,5);
        if Shuffled(trial,2)> 0
            fprintf(obj1, ['BURS:NCYC ' num2str(Shuffled(trial,2))]);
            fprintf(obj1, ['FREQ ' num2str(Shuffled(trial,4)) ' HZ']);
```

```
            fprintf(obj1, 'TRIG');
        end
        err=RP2nd.SetTagVal('msTone',Shuffled(trial,3)); %ms of tone
        err=RP1st.SetTagVal('ms4PP',msISI); %ms time from start of any
stim to SES)
        err=RP1st.SetTagVal('ms4ES',Shuffled(trial,1));

        RP2nd.SoftTrg(1);
        RP1st.SoftTrg(1);
        pause(max(time)); %round up to second past end of data
collection
        accel=RP1st.ReadTagV('dataout', 0, maxPoints); %read the data
        subplot(2,1,1);
        plot(time,accel);
        start=floor(msISI/1000*fs) + ADCdelay;
        resp=accel(start+1:(start+pnts2Meas));
        temp=max(max(resp),-min(resp));
        maxmax=max(temp,maxmax);
        if temp>= 10
         nOvld=nOvld+1;
        end
        subplot(2,1,2);
        resp=resp-mean(resp);
        plot(1:pnts2Meas,resp);
        RMS=norm(resp)/sqrt(pnts2Meas);
        disp(['block ' num2str(block)...
              ' trial ' num2str(trial)...
              ' ms4ES= ' num2str(Shuffled(trial,1))...
              ' cyPerBuzz= ' num2str(Shuffled(trial,2)) ...
              ' Hz of Buzz= ' num2str(Shuffled(trial,4)) ...
              ' msTone= ' num2str(Shuffled(trial,3)) ...
              ' msISI= ' num2str(Shuffled(trial,5)) ...
              ' RMS is ' num2str(RMS) ...
              ' p-p is' num2str(max(resp)-min(resp))])

        %save the data: block trial PP startle RMS VPtP
        data(tR,1)=block;
        data(tR,2)=trial;
        data(tR,3)=Shuffled(trial, 1);
        data(tR,4)=Shuffled(trial, 2);
        data(tR,5)=Shuffled(trial, 3);
        data(tR,6)=Shuffled(trial, 4);
        data(tR,7)=Shuffled(trial, 5);
        data(tR,8)=RMS;
        raw(tR,1:maxPoints)=accel(1:maxPoints);

        iti= rand()*(ITIs(2)-ITIs(1)) +ITIs(1);
        pause(iti)
        tR=tR+1;
    end
end %end of blocks
finish=rem(now,1)
clock
pause off
RP1st.Halt;
RP2nd.Halt;
```

```
if saveIt
    time=fix(clock);
    comment2=input('enter any final comment(or none)then Enter', 's');
    save (filename)
    xlswrite([filename 'data.xls'],data);
    %xlswrite([filename 'raw.xls'],raw');
    diary ([filename '.PPI'])
end

if ~debug
    useit1=(data(:,3)==ms4ES & data(:,4)==0 & data(:,5)==0);  %full
startle
    ASRc=mean(data(useit1,8));
    useit0=(data(:,3)==0 & data(:,4)==0 & data(:,5)==0);  %baseline, no
PP nor ES
    base=mean(data(useit0,8));
    [~,p] = ttest2(data(useit1,7),data(useit0,7));
    disp(['ASRc = ' num2str(ASRc) ' above base of ' num2str(base) ' p =
' num2str(p)])
%     useit=(data(:,3)==0 & data(:,4)==5 & data(:,5)==0);
%     strtlByBuzz=mean(data(useit,7));
%     [~,p] = ttest2(data(useit,7),data(useit0,7),'tail','right');
%     disp(['startles by buzz alone (no SES) = ' num2str(strtlByBuzz) '
p>baseline= ' num2str(p)])
%     useit=(data(:,3)==0 & data(:,4)==0 & data(:,5)==50);
%     strtlByTone=mean(data(useit,7));
%     [~,p] = ttest2(data(useit,7),data(useit0,7),'tail','right');
%     disp(['startles by tone alone (no SES) = ' num2str(strtlByTone) '
p>baseline= ' num2str(p)])
    PPI=NaN(5);
    pPI=NaN(5);
    for i=1:12
        switch i
            case 1
                buzz=2; tone = 4; t=2; b=2;
            case 2
                buzz=4; tone = 9; t=3; b=3;
            case 3
                buzz=9; tone = 25; t=4; b=4;
            case 4
                buzz=10; tone = 45; t=5; b=5;
            case 5
                buzz=2; tone=0; t=1; b=2;
            case 6
                buzz=4; tone=0; t=1; b=3;
            case 7
                buzz=9; tone = 0; t=1; b=4;
            case 8
                buzz=10; tone = 0; t=1; b=5;
            case 9
                buzz=0; tone = 4; t=2; b=1;
            case 10
                buzz=0; tone=9; t=3; b=1;
            case 11
                buzz=0; tone=25; t=4; b=1;
            case 12
                buzz=0; tone=45; t=5; b=1;
```

```
            otherwise
                disp('error in switch statement')
        end
        useit=(data(:,3)==ms4ES & data(:,4)==buzz & data(:,5)==tone);
        ASRp=mean(data(useit,8));
        PPI(b,t)=1-(ASRp/ASRc);
        temp=1-(data(useit,8)/ASRc);
        [~,pPI(b,t)]=ttest(temp,0,'tail','right');
        disp(['ASRp for buzz= ' num2str(buzz) ' and tone = '
num2str(tone) ' is ' num2str(ASRp) ' for PPI = ' num2str(PPI(b,t))])
    end
    PPI
    pPI
%     useit=(data(:,3)==ms4ES & data(:,4)==5);
%     temp=1-(data(useit,7)/ASRc);
%     [~,p]=ttest(temp,0,'tail','right');
%     disp(['PPI to 50ms buzz = ' num2str(mean(temp)) ' p= '
num2str(p)])
    figure
    plot(PPI','s','MarkerSize',10)
    hold on
    plot(nanmean(PPI))
    figure
    plot(PPI,'o','MarkerSize',10)
    hold on
    plot(nanmean(PPI'))
end
diary off
if saveIt
    h=gcf; %get handle of Fig 2
    saveas(h,filename,'fig')
end
disp ([' # overloaded recordings = ' num2str(nOvld) ...
       ' maximum input voltage = ' num2str(maxmax)])

disp(['done after ' datestr(finish-start,'HH:MM:SS')]);
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