Pre-pulse inhibition assessment of sound localization in mice: Methodological, functional, and genetic considerations

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Pre-Pulse Inhibition Assessment of Sound Localization in Mice: Methodological, Functional, and Genetic Considerations

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A dissertation submitted to the Graduate Faculty of

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In

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Introduction to the Reader

This dissertation is divided into three parts. The first part is in manuscript format in preparation for publication and includes an introduction, methodology, results, discussion and conclusion. The second part of this dissertation is an expanded literature review for the purpose of establishing a background for this research. The third part includes an appendix to highlight supplemental findings.
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Abstract

Sound localization is an important aspect of hearing, and our sequencing of the mouse genome makes it an increasingly attractive mammalian model with its capacity for transgenics and conditional knockouts. One way to study sound localization in mice is through pre-pulse inhibition (PPI) – an unconditioned reflex where a weak pre-stimulus inhibits the subsequent response to a louder startling stimulus. To study sound localization, the pre-stimulus can be a soft, ongoing sound that switches between two speakers. If the mouse hears the change in location, the acoustic startle response will decrease. We measured PPI in C57BL/6J, BALB/c, and wildtype EphA4 mice from a 180° speaker-swap in three different test chambers. We conclude that test chamber is critically important in the behavioral assessment of sound localization. Test chambers having free sound penetration are essential for behavioral studies of sound localization. Depending on the chamber, we observed 17%, 33% and 100% of responsiveness noted in a previously published study. Our experiment emphasizes the importance of standardizing test chambers to make comparisons within and across sound localization studies using PPI.
Part 1: Manuscript

Effect of Test Chamber on Sound Localization in Mice (Experiment 1)

Introduction

Murine Model for Auditory Research

The mouse has become a widely used model for mammalian hearing due to anatomical, physiological and genomic similarities with humans. Regarding genomic similarity, approximately 99% of genes in mice have a human counterpart, with many of these homologous regions being directly aligned (Avraham, 2003). With the characterization of the mouse genome, it is a particularly suitable model system for manipulation studies that seek to test the importance of certain genes/proteins for various aspects of hearing. Certain mouse strains exhibit a spectrum of naturally occurring hearing impairments, in addition to genetically engineered impairment models, making them ideal for hearing research (Avraham, 2003; Erway, Willott, Archer, & Harrison, 1993; Hof & Mobbs, 2001; Johnson, Erway, Cook, Willott, & Zheng, 1997; Ohlemiller, 2006; Willott et al., 1998; Zheng, Johnson, & Erway, 1999).

Sound Localization

The ascending auditory pathway includes the auditory nerve, cochlear nuclei, superior olivary complex, lateral lemniscus, inferior colliculus, medial geniculate body and auditory cortex. There are three cues essential for sound localization via this pathway: interaural time differences (ITDs), interaural level differences (ILDs), and spectral processing. ITDs rely on the precise timing of the arrival of a signal at the two ears. ILDs rely on the intensity differences at the two ears from the head shadow effect. Spectral cues result from frequency modifications of a signal arriving at the eardrum.
from the head-related-transfer-function. Mice rely on ultra-high frequency hearing for sound localization. In addition, the head size of the mouse is small. For these reasons, ITDs are of little use as a sound localization cue. ITDs, which are primarily useful for low frequency localization tasks, become increasingly small as head size decreases because larger wavelengths bypass the head and arrive with similar timing at the opposite ear (Ehret & Dreyer, 1984).

*Acoustic Startle Response (ASR) and Pre-pulse Inhibition (PPI)*

The acoustic startle response (ASR) is a short latency muscle reflex that is elicited by an unexpected, loud signal. The ASR has been used to study complex neurobiological processes (Acri, Grunberg, & Morse, 1991; Johansson, Jackson, Zhang, & Svensson, 1995; Ludewig, Geyer, Etzensberger, & Vollenweider, 2002; Morgan, Grillon, Southwick, Davis, & Charney, 1996). The ASR holds several advantages over other behavioral methods, such as operant conditioning, in that it does not require prior experience, training, or reinforcement (Ison & Hoffman, 1983; Schmajuk & Larrauri, 2005; Sweardlow, Braff, & Geyer, 2000).

The ASR is used extensively in the behavioral assessment of sensorimotor performance, and experimental conditions can be altered to measure their subsequent effects on the ASR. An example of acoustic startle response modification is pre-pulse inhibition (PPI). PPI occurs when a weak pre-stimulus inhibits the response to a subsequent startle stimulus. PPI circuitry is thought to involve the cochlear nuclei, superior olivary complex, lateral lemniscus, inferior colliculus, superior colliculus, pedunculopontine tegmental and caudal pontine reticular nuclei. Lesions of the inferior colliculus (IC) significantly affect pre-pulse inhibition to acoustic stimuli, showing that
the IC makes an important contribution to the modification of the startle response (Fendt, Li, & Yeomans, 2001; Li, Korngut, Frost, & Beninger, 1998).

Pre-pulse inhibition (PPI) is a proven, useful method for analyzing hearing in mice. PPI has been used to study hearing loss, hearing loss induced plasticity, frequency and temporal processing, tinnitus assessment, auditory system development, aging, genetic effects, and sound localization (Carlson & Willott, 1996; Fitch, Threlkeld, McClure, & Peiffer, 2008; Ison, Agrawal, Pak, & Vaughn, 1998; Longenecker & Galazyuk, 2012; Ouagazzal, Reiss, & Romand, 2006; Paylor & Crawley, 1997). However, to our knowledge there has been no previous report of genetic effects on sound localization in mice using PPI.

Sound localization has been studied in mice using pre-pulse inhibition from a 180° azimuth speaker swap at varying interstimulus intervals (ISIs) (Allen & Ison, 2010). Robust PPI responses were measured at ISIs between 10-100 ms, with a peak (maximum decrement of ASR) of 0.6 at 50 ms (Allen & Ison, 2010). The present study aims to repeat this same paradigm, obtain similar results in different test chambers, and ultimately test sound localization in mice with Eph/ephrin mutations.

**Statement of the Problem**

Little research is available on the behavioral study of sound localization using a pre-pulse inhibition experimental paradigm. To our knowledge, there is no published replication of Allen & Ison (2010)’s speaker swap PPI study of sound localization in mice. The goal of the current experiment is to better understand the methodological and functional considerations of using this paradigm for sound localization analysis and to
establish a standard to be used in subsequent experimentation in a variety of control and mutant strains.
Materials and Methods

Subjects

Control assessments consisted of C57BL/6J mice (n = 9) and well as wildtype animals (EphA4+/+, see below for genotyping procedures) from our EphA4 colony (n=11). These mice were tested between the ages of 24 and 90 days. All mice were tested twice, approximately two weeks apart. The C57BL/6J strain is known to have progressive hearing loss; therefore, no mouse of this strain or bred on this background was tested after 8 months of age (Hof & Mobbs, 2001; Johnson et al., 1997; Willott, Carlson, & Chen, 1994; Zheng et al., 1999).

In addition, BALB/c mice were tested in what will be termed Chamber #2. The BALB/c strain was used to determine if our poor results in Chamber #2 might have been due to some unexpected strain- or age-related effect. BALB/c mice were tested at two ages: young (n=6; tested between 29 and 34 days of age) and old (n=6, tested between 185 and 200 days of age). Mice of the BALB/c group were tested between one and three times.

All mice were group-housed (approximately 4 mice per cage) in a BioZone MiniSmart Rack System in a controlled environment. The James Madison University Institutional Animal Care and Use Committee (IACUC) gave approval for all practices prior to data collection.

Genotyping procedures

EphA4\textsuperscript{lacZ} mouse colony was maintained on a C57/BL6J background strain. Tail samples (~2 mm) and ear tagging, for identification, were performed under light anesthesia (3% isofluorane). Tails were denatured for 1 hour at 98°C in tail denature
buffer (25 mM NaOH, 0.2 µM EDTA). Post-digestion, samples were neutralized with neutralization solution (40 mM Tris-HCl, pH 5.5). PCR amplification (94°C 30 s; 31 cycles: 94°C, 30 s, 61°C, 30 s, 72°C, 2 min; one final elongation at 72°C, 10 min) was performed with JumpStart™ REDTaq® ReadyMix™ Reaction Mix (Sigma-Aldrich P0982) and the following primer sequences: EphA4-forward 5’-
AGACATTCCAGAAGGGAGTCAG-3’; EphA4-reverse 5’-
ATAGACAGGACACAGTGAAGCCAC-3’; lacZ-forward 5’-
GCACCGATCTAGTTGAAGACATC-3’; lacZ-reverse 5’-
CACGCCATACAGTCCTCTC3ACATC-3’. Gel electrophoresis of PCR products with EphA4-forward and EphA4-reverse primer set resulted in a 376 base pair band (wild-type), and the lacZ-forward and lacZ-reverse primer set produced a 729 base pair band (mutant Eph-A4lacZ allele).

Apparatus and Stimuli

Mice were tested in three different test chambers. The first two chambers were securely glued to the 200 x 125 x 4 mm Plexiglas base (part 7500-0320) of the SR-LAB™ Startle Response System (San Diego Instruments, Inc.). The plate formed the floor of the third chamber. The plate is supported by four 24 mm ‘standoffs’ in the corners, allowing the plate to flex. The plate is supplied with an unlabeled black accelerometer, 50 mm diameter by 12 mm width, affixed beneath the plate.

The first test chamber supplied by San Diego Instruments was made out of clear Plexiglas (37 mm inside diameter, 50 mm outside diameter, 110 mm inside length). The chamber had one 18 x 10 mm oval hole, as well as four 4 mm wide slits, in the top of the cylinder. This will be referred to as Chamber #1.
The second test chamber was locally fabricated from an opaque white PVC pipe (40 mm inside diameter, 48 mm outside diameter, 115 mm in length), with sliding doors modeled after Chamber #1. The chamber had twenty-two 4 mm diameter holes along the top of the cylinder, as well as nine 3 x 15 mm slits along each side (18 slits total). This will be referred to as Chamber #2.

The third test chamber was a Stoetling, Inc. (Chicago IL Cat #51326) adjustable wired chamber. Five 3 mm diameter wires could be adjusted to constrain the mouse. The San Diego Instruments base was inserted beneath the lower wires to form a functional floor. A foam block supported the base, allowing accelerations produced by the startle response to be registered. This will be referred to as Chamber #3.

Test chambers are displayed in Figure 1. All test chambers were set up in the middle of a 2.13 m x 2.13 m Industrial Acoustic sound-attenuating booth. The long axis of each chamber was aligned perpendicular to the line between the two speakers producing the pre-pulse stimulus.
Figure 1. Test chambers used in current experiment: a) Chamber #1; original San Diego Instruments Plexiglas chamber, b) Chamber #2; modified PVC chamber, and c) Chamber #3; Stoetling, Inc. chamber.
The startle stimulus was presented from a Ross Audio Systems TW 30 compression tweeter 15 cm above the test chamber. The startle stimulus was 15 ms, high-pass filtered at 8kHz, rapidly gated broadband noise presented at 120 dB SPL. Calibration of the broadband noise startle stimulus showed 120 dB SPL energy within a 768 Hz to 50 kHz band. The startle stimulus was generated by a Tucker Davis Technology Real-Time Processor, TDT RP2.1 running at 100k samples/s, and amplified by a Crown XLS202 amplifier. The pre-pulse stimulus was presented via two Tucker Davis Technology ESI compression tweeters, placed 180° apart, each located 25 cm from the sides of the test chamber. The pre-pulse stimulus was a continuous noise, high-pass filtered at 4 kHz (1 kHz to 100 kHz bandwidth = 70 dB SPL +/- 1 dB SPL) produced at 200k samples/s. Calibration of both startle and pre-pulse stimuli was performed with an Agilent 35670A Spectrum Analyzer, ¼ ‘’ Bruel & Kjaer 4939 microphone, and Listen, Inc. Sound Connect amplifier.

The voltage recorded from the accelerometer was low-pass filtered at 1 kHz and amplified 100 times by a Krohn-Hite model 3343 and input to a TDT-RP2.1. Input was digitized at 100 kHz for 100 ms at the time of startle stimulus presentation.

**General Procedures**

Sound localization in this experiment was measured using a pre-pulse inhibition (PPI) behavioral test method. The pre-pulse stimulus was an instantaneous 180° change in location of the 70 dB SPL broadband noise. The test procedure used in this experiment is the same as in previous studies (Allen & Ison, 2010; Liuzzo, Gray, Wallace, & Gabriele, 2014). Thirteen interstimulus intervals (ISIs) (1, 2, 5, 10, 20, 30, 40, 50, 60, 100, 150, 200 and 300 ms) were randomly presented with two no-prepulse and one no-
startle control trials to form a block of 16 trials, with a random inter-trial interval between 1.5 and 2.5 seconds. These blocks were repeated 11 times for a total of 176 total trials. Testing was completed in approximately one hour.
**Results**

We show no effect of repeated testing, age, or group (C57BL/6J vs. BALB/c vs. EphA4+/+) of mice. We then pooled all mice tested and quantified a significant effect of test chamber.

There was no effect of test time using a repeated measures ANOVA, with 26 within-subjects factors (13 ISIs and 2 test times) (p=.73). A marginally significant and uninterpretable interaction was ignored: a multivariate ISI*time*cage interaction (p=.044 by Pillai’s Trace and >.05 by Hotelling’s or Wilk’s tests). Thus, the PPIs for each of the 13 ISIs were averaged over the first and second test for each mouse.

BALB/c mice (n=12) were tested in Chamber #2 as a control to confirm whether the unexpectedly poor responsiveness was due to strain considerations with the C57BL/6J and EphA4 mice. BALB/c mice were tested between one and three times each, so responses at each ISI were averaged for each mouse. There was no significant difference between old and young BALB/c mice (p=.33); therefore, all BALB/c mice were pooled.

We found no effect between the three groups of mouse controls. In Chamber #1, ‘group’ was the comparison of C57BL/6J controls and EphA4+/+. In Chamber #2, ‘group’ included C57BL/6J and EphA4+/+, as well as the BALB/c strain. In Chamber #3, only C57BL/6J controls were tested; therefore, there is no analysis of ‘group’ in this test chamber. Repeated measures ANOVAs using 13 averaged ISI as the within-subjects factor and ‘group’ (defined above) as the between-subjects factor determined that all mice behaved the same in each test chamber. There was no effect of ‘group’ in Chamber #1 (main effect at p=.992, ISI*group at p=.43). There was no effect of ‘group’ in
Chamber #2 (main effect at $p=.85$, ISI*group at $p=.49$). With no effect of group, all mice were pooled (C57BL/6J, EphA4$^{+/+}$, and BALB/c).

Next, and most importantly, we evaluated the responsiveness of all mice in the different test chambers. Figure 2 shows that the responsiveness in the three chambers was significantly different ($F_{2,29}=35$, $p<.001$). The effect size, $\eta^2=.709$, is large (Cohen, 1988) having defined a ‘large’ eta-squared as .14 and ‘medium’ as .06).

![Graph of Pre-pulse inhibition of the acoustic startle response against ISI for pooled mice in the three different test chambers. Curve from Allen & Ison (2010) is plotted on the graph for comparison.](image)

Figure 2. Pre-pulse inhibition of the acoustic startle response against ISI for pooled mice in the three different test chambers. Curve from Allen & Ison (2010) is plotted on the graph for comparison.
Results in Chamber #3 closely replicate the data of Allen & Ison (2010) as shown in Figure 2. The ISI*cage interaction was not significant (p=.08), meaning that the curves of PPI vs. ISI in the three cages are parallel. Parallel curves suggest that results obtained in Chambers #1 and #2 are attenuated versions of the maximal response. Figure 3 displays a close fit of data to fractions data from Allen & Ison (2010). Chamber #2 produced responsiveness that was attenuated by a third. Chamber #1 produced responsiveness that was attenuated by a sixth. Therefore, we can conclude that the three test chambers replicated the quadratic function of PPI vs. ISI, only with decreased responsiveness in sub-optimal chambers.

Figure 3. Graph showing close fit of our data from Chamber #1 and Chamber #2 to fractions of Allen & Ison (2010) data.
Discussion

Pre-pulse Inhibition and Sound Localization

Pre-pulse inhibition (PPI) of the acoustic startle response (ASR) using a pre-pulse cue of 180° azimuthal change in sound location is useful in the behavioral study of sound localization in mice. We were able to extend the experimental paradigm to the C57BL/6J, BALB/c, and wildtype EphA4 animals. We see a strong PPI in Chamber #3, with a peak response of 0.52 at an ISI of 20 ms. Even in the test chamber that generated the least responsiveness (Chamber #1), there is still evidence of localization (significant quadratic within-subjects contrast of ISI; $F_{1,10}=17.7; p=.002$). All three chambers showed similar PPI vs. ISI curves, with data from Chamber #3 near replicating that of a previous study (Allen & Ison, 2010). Thus, we can conclude that sound localization ability can be measured using a pre-pulse inhibition experimental paradigm. The immediate change in sound location can be used as a pre-pulse cue, with a decrement in startle response being indicative of localization ability.

Effect of Test Chamber

An important conclusion that can be drawn from these data is a significant difference in the results from the three test chambers. Other experimental conditions (i.e. pre-pulse intensity, ISI, etc.) have been shown to affect pre-pulse inhibition. However, to our knowledge, test chamber has not been investigated as a factor affecting PPI. Depending on test chamber, our data showed 17%, 33% and 100% of responsiveness noted in a previously published study. Thus, the test chamber is critically important when measuring sound localization abilities in mice using a PPI behavioral paradigm. The methods of Allen & Ison (2010) say: “One mouse was tested at a time while confined in
an aluminum wire cage, 5 cm wide, 7 cm long, and 4 cm high, having free sound penetration. The mouse was further restricted by adjustable wire combs oriented with the long dimension of the cage, which lightly pressed against its sides.” The test chamber used in that study is not commercially available.

We presume that the poor responsiveness in Chamber #1 was due to having only one hole and a few slits atop the chamber. Chamber #1 is unlikely to have “free sound penetration,” especially from the sides. Chamber #2 would likely have improved sound penetration due to having many holes and slits on the sides. Furthermore, both Chambers #1 and #2 (with inside diameter of 37 mm and 40 mm) allow the mouse to move its head off the desired axis for maximal perception of a change in sound location. Chamber #3, in contrast, constrained the mouse to an appropriate axis, 90° from each pre-pulse speaker. Chamber #3 is also more transparent to sound due to having only five small horizontal wires and no solid wall around the mouse.
Conclusions

An important conclusion from this study is that experimental conditions using a pre-pulse inhibition paradigm must be carefully considered when studying sound localization. Our experiment points to the test chamber as being critically important to pre-pulse inhibition responsiveness. Therefore, it may be necessary to use a standardized test chamber when studying sound localization. An inappropriate test chamber may prevent researchers from being able to find significant differences between experimental groups. The use of different test chambers among studies for sound localization will also make it difficult to compare results across studies.
Effect of EphA4 Mutation on Sound Localization (Experiment 2)

Introduction

Eph/ephrin Signaling

Eph proteins and their ligands, called ephrins, are known to be the largest family of receptor tyrosine kinases. There are two classes of Eph receptors: A and B. EphA receptors bind ephrin-A ligands and EphB receptors bind ephrin-B ligands, with the exception of the EphA4 receptor which also has strong affinities for ephrin-B2, and –B3. Eph-ephrin signaling is bidirectional, meaning that signaling can involve forward (ephrin-to-Eph) or reverse (Eph-to-ephrin) mechanisms. Eph proteins are responsible for cell migration and the guidance of axons during auditory development (Cramer & Gabriele, 2014; Cramer, 2005).

Of particular interest is the expression of EphA4 in auditory brainstem nuclei (Cramer, 2005). EphA4 expression appears to be steeply graded in the dorsal and ventral cochlear nuclei during the first postnatal week in the mouse. Expression of EphA4 is also graded in the inferior colliculus during this developmental period, but becomes more uniform by postnatal day 18 (Gabriele et al., 2011). The disruption of EphA4 results in decreased topographic spread of cells in the brainstem (Miko, Nakamura, Henkemeyer, & Cramer, 2007). Thus, Eph44 proteins are seemingly necessary for the formation of tonotopic auditory projections. Misexpression or disruption of EphA4 also leads to a significant increase in aberrant ipsilateral and contralateral projections, indicating that EphA4 proteins are responsible for auditory axonal guidance to the appropriate target regions during the development of the auditory system (Allen-Sharpley, Tjia, & Cramer, 2013; Cramer, Bermingham-McDonogh, Krull, & Rubel, 2004; K. J. Huffman & Cramer, 2007).
The central nucleus of the inferior colliculus (IC) is important for sound localization due to its precise tonotopic organization and sharp tuning characteristics. The neurons of the central nucleus receive crossed and uncrossed projections, hence, responding to both binaural and monaural localization cues (K. A. Davis, Ramachandran, & May, 2003; Malinina & Vartanyan, 2004). Sound localization performance is significantly poorer with lesions to the pathways of the IC, providing evidence for its role (K. A. Davis et al., 2003). Since EphA4 has been shown to play a role in the establishment of certain auditory brainstem circuits, we are interested in studying whether EphA4 mutation affects sound localization abilities.

**Statement of the Problem**

There is, to our knowledge, no study focusing on the role of EphA4 mutation on sound localization. The goal of the current experiment is to use pre-pulse inhibition to analyze the behavioral effects of EphA4 mutation on sound localization.
Materials and Methods

Subjects

The control group in this study included C57BL/6J mice (n=9). The experimental group in this study included EphA4\textsuperscript{lacZ} mutants bred on a C57BL/6J background (n=18, 11 wildtype, 4 heterozygous, and 3 homozygous).

All mice were tested between the ages of 29 and 127 days. Control and experimental groups were tested twice, approximately two weeks apart.

Genotyping procedures

Genotyping procedures were the same as those discussed in Experiment 1.

Apparatus and Stimuli

Apparatus and stimuli were the same as discussed in Experiment 1, with the exception that Chamber #3 was not utilized.

General Procedure

Testing procedure was the same as that discussed in Experiment 1.
Results

There was no effect of mutation in Chamber #1 (main effect of mutation at p=.53, ISI*mutation interaction at p=.52). However, the insignificant effect of mutation is expected given the minimal response of the control mice as seen in Figure 2 (above in Experiment 1); thus, a “floor effect” is observed with a limited range below that of the control mice to show a decrement in responsiveness. While there was a minor but significant response of the control mice in Chamber #1 (p=.002) as described above in Experiment 1, there was no significant response among the heterozygous (p=.20) or homozygous (p=.39) mutants (as evaluated by a quadratic effect of ISI on PPI, the most significant effect in other analyses).

A significant interaction of ISI*mutation was measured in Chamber #2 (F_{12.9,174}=1.8, p=.035). There was a marginal main effect of mutation (p=.069), which is reasonable given the expected curve effect of PPI vs. ISI. The significant interaction of ISI*mutation illustrates that the effect of mutation is evident at some, but not all, ISIs as seen in Figure 4. There was no reliable responsiveness among the homozygous mutants (p>.25). However, this may be due to overall decreased sensitivity in homozygous EphA4 mutants (Liuzzo et al., 2014).
Figure 4. Graph illustrating the effect of EphA4 genotype on pre-pulse inhibition of the acoustic startle response to a 180° speaker swap in Chamber #2.
**Discussion**

*Sound Localization*

The present experiment indicates that a pre-pulse inhibition experimental paradigm is useful for studying sound localization and can be used to study effects of mutations on that response. Under one testing condition (Chamber #2), EphA4 mutation had a significant effect on sound localization ability. Similar effects were observed under another condition (Chamber #1), but the effect of mutation failed to reach significance due to poor responses in the control group. As such, sound localization abilities of control and experimental groups can be analyzed and compared.

*Eph/ephrin Signaling*

Growing evidence reveals that EphA4 mutation affects tonotopic patterning in auditory brainstem nuclei and appropriate ipsilateral and contralateral axon guidance. Given that sound localization relies heavily on precise auditory projections and the integration of input from both sides of the brain, we might anticipate that EphA4 mutants show poorer performance compared to controls. From these data, we see that homozygous, but not heterozygous, EphA4 mutations appear to affect sound localization. Heterozygous EphA4 mice, with only partial alteration in the EphA4 protein, showed similar behavioral responses compared to controls. Homozygous EphA4 mice, with EphA4 protein that is incapable of reverse signaling, showed no significant PPI, providing evidence of poorer localization performance compared to the other groups. These results provide evidence for the behavioral consequences of EphA4 mutation to supplement published anatomical studies.
Conclusion

From this experiment, we conclude that a pre-pulse inhibition paradigm can be used to investigate how developmental processes such as Eph/ephrin signaling influence auditory behavior. We found that homozygous, but not heterozygous, EphA4 mutation affects sound localization. Under appropriate experimental conditions, it appears that many genetic mutations can be investigated for their behavioral effects using PPI in mice.
Part II: Extended Literature Review

Murine Model for Auditory Research

The mouse is a widely used animal model for human audition. The anatomical and physiological similarities between the mouse and human make the mouse a useful animal model for hearing research. In addition, the mouse is advantageous in the study of auditory system development in that, unlike most animals, inner ear structures are not mature at birth. The tunnel of Corti begins to open and nerve fibers can be seen running through by postnatal day 4. By postnatal day 8, hair cells are adult-like in appearance and configuration, with the outer hair cells being cylindrical and inner hair cells being ovoid in shape (Mikaelian & Ruben, 1965). The stria vascularis has little blood supply at birth; however, this structure increases in thickness and vascularity and becomes adult-like by postnatal day 8. The basilar membrane, important for cochlear macromechanics, has a normal appearance by postnatal day 10. Efferent nerve fibers appear throughout the cochlea by postnatal day 10 (Kikuchi & Hilding, 1965). Studies show that hearing function begins with the appearance of efferent nerve fibers, and cochlear and nerve potentials can be recorded between postnatal days 9 and 11 (Kikuchi & Hilding, 1965). The external and middle ear are among the last auditory structures to develop and are fully formed by postnatal day 12 (Mikaelian & Ruben, 1965).

Genetic and environmental manipulations in the mouse have improved our understanding of their effects in humans. Approximately 100 mutations resulting in auditory abnormalities in the mouse have allowed us to better understand various etiologies of deafness in humans (Zheng et al., 1999). The mouse has significantly contributed to the understanding of age-related and noise-induced hearing loss in humans.
(Ohlemiller, 2006). For example, the AHL gene, which was first discovered in the C57BL/6J strain, and subsequently nine other strains, has contributed to our understanding of age-related hearing loss (Johnson et al., 1997; Johnson, Zheng, & Erway, 2000). Another example is the identification of the Pou4f3 gene in the mouse, which led to the discovery of the human gene for a form of non-syndromic progressive hearing loss (Avraham, 2003). Table 1 shows genes for which both a mouse and human form of deafness exists.

<table>
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<th>Human Gene</th>
<th>Protein</th>
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<td>Connexin 20</td>
<td>2</td>
<td>(Cohen-Salmon et al., 2002)</td>
<td></td>
</tr>
<tr>
<td>DFNB3/DFNA11</td>
<td>MYO7A</td>
<td>Myosin VII</td>
<td>9</td>
<td>(Gibson et al., 1996)</td>
<td></td>
</tr>
<tr>
<td>DFNB3</td>
<td>MYO5A</td>
<td>Myosin X</td>
<td>3</td>
<td>(Probst et al., 1998)</td>
<td></td>
</tr>
<tr>
<td>DFNB3</td>
<td>TME</td>
<td>Transmembrane inner ear (Tmcx)</td>
<td>2</td>
<td>(Miimoto, et al., 2002)</td>
<td></td>
</tr>
<tr>
<td>DFNB7/B1/A38</td>
<td>TMC1</td>
<td>Transmembrane, cochlear expressed 1</td>
<td>10</td>
<td>(Kurina et al., 2002; Vyasoo et al., 2002)</td>
<td></td>
</tr>
<tr>
<td>DFNB12</td>
<td>CDH23</td>
<td>Cadherin 23</td>
<td>1</td>
<td>(Di Perna et al., 2001; Wilson et al., 2001)</td>
<td></td>
</tr>
<tr>
<td>DFNB38</td>
<td>ESPN</td>
<td>Espin</td>
<td>2</td>
<td>(Nix, Rutter, Rutter, Grifith, Friedman, &amp; Willcox, 2002)</td>
<td></td>
</tr>
<tr>
<td>DFNB37/A22</td>
<td>MYO6</td>
<td>Myosin VI</td>
<td>3</td>
<td>(Avraham et al., 1997)</td>
<td></td>
</tr>
<tr>
<td>DFNA15</td>
<td>POLG</td>
<td>Sex-linked fidget (Spf); Polg5 knock-outs</td>
<td>2</td>
<td>(Avraham et al., 1997; Tcheng et al., 1997)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Avraham², 1997; Tcheng et al., 1997)</td>
</tr>
</tbody>
</table>

Table 1. Genes for which both a mouse and human form of deafness exists (Avraham, 2003).
The C57BL/6J and BALB/c strains were used in the current study. The C57BL/6J strain begins to show signs of hearing loss by 5-6 months of age, with evidence of severe high frequency hearing loss by 1 year of age (Johnson et al., 1997; Willott et al., 1994). Studies have shown that C57BL/6J mice have normal ABR thresholds at 33 weeks of age (8 months) (Zheng et al., 1999). BALB/c mice show a time course of progressive hearing loss similar to the C57BL/6J strain, with some variability among substrains (Hof & Mobbs, 2001). Due to the progression of hearing loss, it was critical that we not test any mice in this study over 8 months of age.

*Acoustic Startle Reflex and Pre-pulse Inhibition*

The acoustic startle reflex is a short latency muscle reflex elicited by an intense acoustic stimulus. The acoustic startle reflex has been particularly valuable in the study of animal behavior. Substantial literature exists detailing the history of the ASR and its uses (Ison & Hoffman, 1983). The majority of studies that look at the neural circuit of the ASR identify the auditory nerve, ventral cochlear nucleus, lateral lemniscus, ventral nucleus of the reticular formation, reticulospinal tract via the medial longitudinal fasciculus, and spinal cord interneurons and motor neurons as the primary synaptic relays (Figure 5) (M. Davis, Gendelman, Tischler, & Gendelman, 1982; Leumann, Sterchi, Vollenweider, Ludewig, & Früh, 2001; Yeomans & Frankland, 1995).
Inhibition of the acoustic startle reflex is known as pre-pulse inhibition (PPI). PPI is a behavioral measure of sensorimotor gating (Koch, 1999; Leumann et al., 2001; Swerdlow, Geyer, & Braff, 2001). PPI is the phenomenon by which a weaker stimulus presented before a startle stimulus reduces the magnitude of the acoustic startle reflex. PPI has been used to study the effects of genetic, pharmacological, and environmental manipulations. PPI as a research tool is valuable in that it 1) is easily quantified and measurable across species and throughout development, 2) provides a means to test and compare large numbers of subjects in a relatively short amount of time, 3) offers the ability to link neurophysiologic manipulations with resulting behavior and 4) holds
advantages over operant conditioning in that it does not require learning, training or reinforcement (Schmajuk & Larrauri, 2005; Swerdlow et al., 2000).

The primary neural circuit for PPI includes the inferior colliculus, superior colliculus, pedunculopontine tegmental nucleus, and caudal pontine reticular nucleus (Figure 6) (Fendt et al., 2001; Koch, Lingenhöhl, & Pilz, 1992; Koch, 1999). Of particular importance to our study is the contribution of the inferior colliculus (IC) to the inhibition of the ASR. Lesions of the IC have been shown to increase the magnitude of the ASR and diminish PPI (Fendt et al., 2001). Thus, the IC is a critical structure in providing a means for communication between the auditory system and the PPI neural circuit.

Figure 6. Flowchart showing the neural circuit for pre-pulse inhibition (PPI) (Schmajuk & Larrauri, 2005).
Visual, tactile, and acoustic pre-pulses have all been successfully used in the study of pre-pulse inhibition (PPI). When using acoustic pre-pulses, several factors much be considered. The intensity of both the pre-pulse and startle stimuli affect responses. ASR decreases and PPI increases with increasing pre-pulse intensities (Koch, 1999; Paylor & Crawley, 1997). Maximal PPI is achieved with a pre-pulse duration of 10-20 ms (Koch, 1999). Even the interstimulus interval (ISI), or the time between the presentation of the pre-pulse and startle stimuli, affects the amount of inhibition of the ASR. Strong inhibition occurs with ISIs between 40-150 ms, with maximal PPI responses at 100 ms (Allen & Ison, 2010; Fendt et al., 2001; Ison, McAdam, & Hammond, 1973; Koch, 1999; Yeomans & Frankland, 1995).

The chosen pre-stimulus (or pre-pulse stimulus) in PPI assessment is dependent upon the focus of the research. Several factors affect the ASR and PPI. Variations in acoustic pre-pulse stimuli allow for the measurement of hearing ability such as hearing sensitivity, discrimination ability, and sound localization (Ison & Hoffman, 1983). Sound localization has been studied using a pre-pulse inhibition test procedure with a 180° speaker swap as an effective pre-pulse (Figure 7) (Allen & Ison, 2010).
Figure 7. Figure from Allen & Ison (2010) showing that localization can be evaluated using pre-pulse inhibition, with a 180° swap in sound presentation being an effective pre-pulse stimulus. PPI peak at 0.6 beginning at an ISI of approximately 10 ms and dropping off at an ISI of 100 ms.

Our study incorporates previously used methods to further analyze sound localization using PPI, as well as extend these methods for the comparison of control and mutant groups.

*Sound Localization*

Sound localization is the auditory process of identifying the precise direction of a sound source, and requires the comparison of temporal, intensity and frequency information from the two ears. The pathway for sound localization includes the auditory nerve, cochlear nuclei, superior olivary complex, lateral lemniscus, inferior colliculus, medial geniculate body, and auditory cortex. The timing, intensity, and frequency information of a signal is preserved by the auditory nerve and cochlear nuclei. The
superior olivary complex is the first major structure to receive binaural input after the ascending auditory pathway crosses. The medial superior olive processes temporal differences provided by low frequencies, whereas the lateral superior olive processes intensity differences provided by high frequencies. The lateral lemniscus receives the crossed and uncrossed projections, and thus preserves the binaural representation of the signal. The inferior colliculus (IC) integrates and expands upon the input from lower auditory structures. The medial geniculate body and auditory cortex are responsible for multisensory integration and the concept of auditory space for localization.

The central nucleus of the inferior colliculus (IC) is particularly important for sound localization due to its precise tonotopic organization and sharp tuning characteristics. The neurons of the IC respond to both binaural and monaural localization cues (K. A. Davis et al., 2003; Malinina & Vartanyan, 2004). Sound localization performance is significantly poorer with lesions to the pathways of the IC, providing evidence for its role in this complex auditory process (K. A. Davis et al., 2003).

Sound localization relies on three cues, interaural time differences (ITDs), interaural level differences (ILDs) and monaural spectral processing. Humans use all three cues to identify a sound source. Mice, on the other hand, use two of the three sound localization cues. The mouse’s head is too small to make use of ITDs, so instead the mouse uses ILDs and spectral cues for sound localization (Ehret & Dreyer, 1984).

Eph/ephrin Signaling

Eph proteins and their ligands, called ephrins, are known to be the largest family of receptor tyrosine kinases. There are two classes of Eph receptors: A and B. EphA receptors bind ephrin-A ligands and EphB receptors bind ephrin-B ligands, with
the exception of the EphA4 receptor which also has strong affinities for ephrin-B2, and – B3. Eph-ephrin signaling is bidirectional, meaning that signaling can involve forward (ephrin-to-Eph) or reverse (Eph-to-ephrin) mechanisms. Eph proteins are responsible for cell migration and the guidance of axons during auditory development (Cramer & Gabriele, 2014; Cramer, 2005).

Effects of Eph/ephrin Signaling on Afferents

Of particular interest is the role of Eph/ephrin signaling in the development of the auditory system, and its function in the formation of the precise auditory circuit necessary for sound localization. Studies have shown that EphA4 is highly expressed in auditory brainstem nuclei (Cramer, 2005). EphA4 expression is steeply graded at postnatal day 3 in both the ventral and dorsal cochlear nuclei and inferior colliculus (Gabriele et al., 2011). Expression gradients are high to low, with evidence of strong expression in the ventromedial, high-frequency regions and faint EphA4 expression in the dorsolateral, low-frequency regions. By postnatal day 18, expression of EphA4 becomes more uniform (Gabriele et al., 2011; Miko et al., 2007).

EphA4 plays a role in the discrete and continuous patterning of auditory projections during development. More specifically, EphA4 is involved in topographic mapping and boundary formation for auditory brainstem circuitry (Allen-Sharpley et al., 2013; Gabriele et al., 2011). Studies have shown that misexpression of EphA4 resulted in a decreased topographic spread of cells in the brainstem (Miko et al., 2007). EphA4 has also been shown to play a role in axon guidance and cell migration. Studies in the chick show that disruption of EphA4 signaling lead to an increase in aberrant ipsilateral and contralateral auditory projections (Cramer et al., 2004; Ho et al., 2009; K. J. Huffman &
In addition, EphA4 mutation resulted in abnormalities to auditory nuclei structure, which is indicative of inaccuracies in cell migration (Cramer et al., 2004).

Effects of Eph/ephrin Signaling on Efferents

As previously discussed, EphA4 signaling is responsible for tonotopic mapping, axon guidance, and cell migration in auditory system. The inferior colliculus (IC) is an important auditory structure, and studies have shown strong expression postnatally in the mouse. The IC is believed to have a connection with the cochlea via the olivocochlear efferent pathway. Studies have shown that stimulating the IC resulted in modifications to the responses from the auditory nerve (R. F. Huffman & Henson, 1990). In addition, the IC contributes to the descending acousticomotor pathway in that lesions of the IC resulted in the absence of the acoustic startle response (R. F. Huffman & Henson, 1990).

Evidence also exists for Eph/ephrin signaling in the development of motor pathways. The motor cortex controls body movements topographically via the corticospinal tract (CST). EphA4 plays a significant role in the topographic mapping of the CST and axon branching in the spinal cord. Disruption of EphA4 signaling results in aberrant axonal projections to the dorsal hindlimb, rather than their correct projections to the ventral hindlimb (Eberhart, Swartz, Koblar, Pasquale, & Krull, 2002). Consequently, EphA4 mutant mice have a hopping gait. This phenotype is more often seen in homozygous EphA4 mutants compared to heterozygous EphA4 mutants (Helmbacher, Schneider-Maunoury, Topilko, Tret, & Charnay, 2000).

EphA4 has been shown to play a part in the development of descending auditory and motor projections. Therefore, we assume EphA4 misexpression would affect multiple
facets of efferent processing. The current study attempts to use the acoustic startle response and pre-pulse inhibition to measure EphA4 mutation on efferent processing.
Abstract

The acoustic startle response (ASR) is used to study hearing – the development, ageing and various complexities of the afferent system. However, it also has an efferent component in that it involves a startle, an unconditioned motor reflex. Mutant mice are used to investigate the effects of various genes on afferent processing. Thus, if the ASR is used to study hearing in mutant mice, there might also be an efferent influence of the mutation(s). In this study, we used classical multidimensional scaling (MDS) to reveal differences among detailed patterns of the startle responses of genetically different mice. Thirty mice in five groups (C57BL/6J, EphA4\(^{+/+}\), EphA4\(^{+/-}\), EphA4\(^{-/-}\), and BALB/c) were tested approximately two times each. Over two million points, digitized from an accelerometer beneath the mice at 100kHz for 20 ms after the onset of a 15 ms, 120 dB broadband startling stimulus were averaged and normalized to constant amplitude. Euclidean distances were calculated for all possible pairs (1770) of these 60 averages and input to MDS. The resulting X-axis coordinates of the MDS solution (the first principal component) were a useful summary of underlying patterns in these data. BALB/c mice had a different pattern of reflex responsiveness than C57BL/6J mice, understandable given that these are inbred strains with many other differences. Of greater interest is that EphA4 mutation, both homozygous and heterozygous, affected the startle response. Genetic differences between strains of mice and between C57BL/6J and EphA4 mutant
mice affected a central pattern generator. EphA4 protein is thus implicated in the establishment of afferent and efferent neural pathways.
Introduction

Efferent Influences on ASR and PPI

The acoustic reflex pathway involves both ascending and descending projections and primarily consists of the ventral cochlear nucleus, lateral lemniscus, reticular formation, reticulospinal tract via the medial longitudinal fasciculus, and spinal cord interneurons and motorneurons (M. Davis et al., 1982; Leumann et al., 2001; Yeomans & Frankland, 1995). The reticulospinal neurons of the caudal pontine reticular formation are particularly important in mediating the acoustic startle reflex by conveying multisensory input into motor output (Koch et al., 1992).

Pre-pulse inhibition (PPI), which reflects a modification to the acoustic startle response, is a measure of sensorimotor gating. PPI is mediated by the inferior colliculus, superior colliculus, pedunculopontine tegmental nucleus (PPTg), laterodorsal tegmental nucleus, substantia nigra, and caudal pontine reticular nucleus (Fendt et al., 2001). PPI has often been used experimentally to study afferent processes; however, what about its use in studying efferent processes?

The inferior colliculus (IC) is of particular interest for its role in modifying the amplitude of the startle reflex (Swerdlow & Geyer, 1993). The IC can be divided into several divisions, with each division consisting of afferent and efferent projections. The central nucleus of the IC (ICC) receives the afferent fibers of the ascending auditory system, as well as sends descending efferent projections to the superior olivary complex and cochlear nuclei. The dorsal nucleus of the IC (ICD) receives descending input from the auditory cortex. The external nucleus of the IC (ICX) receives ascending input, while also sending descending input for acousticomotor responses (R. F. Huffman & Henson,
1990). A descending pathway from the IC to the motor system would allow for a short latency feedback mechanism for the acoustic startle response.

**Role of EphA4 on Efferent System**

EphA4 protein has been proven to play a role in descending auditory and motor processes. In the auditory system, EphA4 is responsible for cell migration and the guidance of axons to appropriate target regions during development (Cramer & Gabriele, 2014). Another important role for EphA4 is the tonotopic organization of auditory brainstem nuclei. Mutation of EphA4 results in inappropriate topographic boundaries in the brainstem (Miko et al., 2007). Thus, we can expect that EphA4 misexpression would affect the efferent systems ability to provide accurate auditory feedback.

In the mouse, EphA4 has also been shown to be involved in the segregation of dorsal and ventral motor axon trajectories, as well as in the topographic organization of motor projections. Disruption of EphA4 signaling results in atypical axonal projections to the dorsal hindlimb, rather than correct projections to the ventral hindlimb (Eberhart et al., 2002). The behavioral phenotype associated with these EphA4 mutations is abnormal gait and posture, the synchronous movement of right and left limbs, and difficulty initiating movement (Helmbacher et al., 2000). These phenotypes are more prevalent in homozygous EphA4 mutants compared to heterozygous EphA4 mutants, with 88% of homozygous mutants showing the abnormal motor behavior compared to only 30% of heterozygous mutants (Helmbacher et al., 2000).

**Statement of the Problem**

The acoustic startle response has been used to study hearing. Mutant mice have been used to study the effects of various genes on afferent auditory processing. However,
there is an important efferent component involved in auditory behavior that is often overlooked. Our goal is to investigate the influence of EphA4 mutation on efferent processing using pre-pulse inhibition of the acoustic startle response. Since we know that EphA4 mutation affects critical auditory and motor structures, we hypothesize that we will see an effect on responses mediated by the efferent system.
Materials and Methods

Subjects

Five groups of mice were used in this study: 1) C57BL/6J (n=6), 2-4) three different genotypes of EphA4\textsuperscript{lacZ} mutants bred on a C57BL/6J background (n=12, 2 wildtype, 4 heterozygous, and 6 homozygous), and 5) BALB/c (n=12).

All mice were tested between the ages of 29 and 200 days. Each group was tested twice, approximately two weeks apart.

Genotyping procedures

Genotyping procedures were the same as those in discussed in Part I.

Apparatus and Stimuli

Apparatus and stimuli were the same as that in Part I; with the exception that only Chamber #2 was utilized.

General Procedure

Testing procedures were the same as those in Part I.
Analysis

(Written by Lincoln Gray; edited by Megan Klingenberg)

Thirty mice were tested in Chamber #2, producing a total of 60 tests (each mouse being tested twice, with a few exceptions). Twenty-two of the 176 trials in each test were of the startling stimulus alone (no-prepulse).

<table>
<thead>
<tr>
<th></th>
<th># of Mice</th>
<th># of Tests</th>
<th># of Startles</th>
<th># of Points</th>
</tr>
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<tbody>
<tr>
<td>C57BL/6J Control</td>
<td>6</td>
<td>12</td>
<td>264</td>
<td>528,000</td>
</tr>
<tr>
<td>BALB/c</td>
<td>12</td>
<td>26</td>
<td>572</td>
<td>1,144,000</td>
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<tr>
<td>EphA4 +/-</td>
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<td>4</td>
<td>88</td>
<td>176,000</td>
</tr>
<tr>
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<td>4</td>
<td>8</td>
<td>176</td>
<td>352,000</td>
</tr>
<tr>
<td>EphA4 +/-</td>
<td>6</td>
<td>10</td>
<td>220</td>
<td>440,000</td>
</tr>
<tr>
<td>Totals</td>
<td>30</td>
<td>60</td>
<td>1320</td>
<td>2,640,000</td>
</tr>
</tbody>
</table>

Table 2. Summary of analysis.

For each of the 60 tests, all 22 of the startle-only responses were averaged. Only the first 20 ms of the startle-only stimuli were used in this analysis. To analyze the intrinsic pattern of the responses, not the overall amplitudes, which might have differed by the weight of the mouse and other factors such as position of the feet relative to the accelerometer, the amplitude of each mouse’s average waveform was normalized from 0 to 1. The data for the next stage of analysis were 60 different vectors of 2000 points with a maximum of 1 and a minimum of 0. Figure 8 shows these vectors for the control (C57BL/6J and EphA4+/+) as well as the mutant (EphA4+/− and EphA4−/−) mice.
Figure 8. Averaged normalized responses of the control and mutant mice.

To examine any differences in the detailed patterns of the startle responses, we calculated the difference between each vector. A Euclidean distance was calculated between all possible pairs of these 60 vectors (=tests, roughly 2 per mouse). There are 1770 pairs of these vectors (\(\binom{60}{2}\)), and thus 1770 distances were calculated. The greater the difference between the averaged normalized recordings from the accelerometer, the greater the calculated distance between them.

Figure 9 explains the Euclidean distance formula. If the response from the accelerometer had only been one point, the distance would have simply been the distance between those two points (actually the square-root of the squared difference). If the response had been two points, the distance would be the length of the line going from one point to the other – the hypotenuse of a triangle. Similarly, if there had been only three points in each averaged trace from the accelerometer, the distance would also have been the length of the line going from one point to the other. We cannot graph points in more than three dimensions, let alone in 2000 dimensions. However, the normalized vectors each had 2000 points, being voltages recorded at a rate of 100 kHz in the first 20 ms after
the startle response. The Euclidian distance is the square root of the sum of the squared difference in volts between the first points plus the squared difference between the second points and so on. If two vectors were identical, the distance between them would be 0. There are no negative distances (as it is a sum of squares). As the responses from the accelerometer diverge, the calculated distance increases. The Euclidean distance is somewhat like a reversed correlation in that a distance of 0 would have a correlation of 1, and low correlations would have higher distances.

![Diagram of Euclidian distances in 1, 2, 3, >3 dimensions.](image)

Figure 9. Calculation of Euclidian distances in 1, 2, 3, >3 dimensions.

These 1770 measures of distance were input to multidimensional scaling (MDS). Simply stated, MDS makes a plot of points, each point represented on test (one averaged normalized vector) such that distances between points are proportional to the input data (1770 calculated Euclidean distances between each of the 60 vectors). The following explanation of MDS has been adapted from Gray (2013).
Multidimensional scaling excels in extracting underlying patterns in variable data. This process is appropriate to reveal what is ‘underneath’ noisy data – when you struggle to understand the ‘bottom line’ in a complex set of numbers. This process is unbiased in that the data tell us what is important. We do not start by presuming to know the important trends in the data. It is often said that a picture is worth a 1,000 words. Information is neither created nor destroyed in the process of making these maps, just transformed from confusing and obfuscated forms into a unified ‘vision’ of the important trends therein.

MDS is easier to describe in the reverse. Suppose we had a map of a set of items, perhaps five large cities in the ‘corners’ of America as seen in Figure 10. Given such a map it would be easy to construct a matrix of distances between all possible pairs of cities. Such a matrix is frequently found at the bottom of travel maps. Only ten distances are necessary, even though a 5 \times 5 matrix would have 25 cells. We assume that the distance from each city to itself is zero, so the diagonal elements are not needed. We further assume that the distance from city A to B is the same as the distance from city B to A. Thus the matrix is symmetrical. All that is needed is the off-diagonal half-matrix of distances. For a map of N points the off-diagonal half matrix would contain what mathematicians write as \( \binom{N}{2} \), the number of possible pairs of N items. This equals \( N(N - 1)/2 \).

![Figure 10. Illustration of MDS.](image)

MDS takes the off-diagonal matrix and from it constructs a map in some stated number of dimensions such that distances in the map are maximally correlated with the input data (Kruskal and Wish 1978; Schiffman et al.1981). If the data were those from the five cities in America, MDS would produce a plot of these points roughly as we see in common wall maps.

Another way to think of the process of MDS is shown in Figure 11. Ten different proximity measures are shown as partially folded ribbons (or they could be springs). These ribbons represent all possible comparisons between five items shown as the squares labeled A–E. The length of the ribbon represents the Euclidean distances that were calculated from the averaged, normalized responses from the accelerometer: longer ribbons for large distances. Think of these ribbons as labeled on each end to represent the two items that were compared. There would be four ends labeled A that would have B–E on the other end; four ends labeled E that would have A–D on the other end, etc. Now suppose that all of the ends labeled A were tied together, all the ends labeled B tied together, etc. What MDS does is find positions of these five knots (A–E) such that the ribbons are perturbed (shortened or lengthened) as little as possible.
If the ASRs in all mice were a monolithic, unchanging reflex, all the vectors would be the same, and all the calculated distances would be near 0. MDS would put all points into a single cloud of closely clustered points as idealized in Figure 12A.

Alternately, if there were even subtle differences in the way mice startled, then MDS is likely to reveal this by points of one group being in a different place on the map compared to another group. Such an idealized result, if genetic differences affected the startle response, is seen in Figure 12B.

Figure 12. Two different idealized versions of MDS results [where B= BALB/c, C= C57BL/6J, and E= EphA4−/−] (A) Represents similar startle patterns across groups, (B) Represents different startle patterns across groups.
Results
(Written by Lincoln Gray; edited by Megan Klingenberg)

Figure 13 shows the MDS result. There is considerable variability, and there are clearly some outlying points, but some patterns can be seen: 1) BALB/c mice, represented by the blue points, are generally in the lower left, and 2) Mutant mice, represented by the green and tan points are towards the right.

![MDS result](image)

Figure 13. MDS result, represented by a 2D plot of the five groups of mice.

The x-axis in any MDS solution is the principal component that explains most of the variance. Thus, the subsequent analyses are simplified to consider only the X-axis coordinates calculated by MDS in order to provide the best possible fit to the input data. Figure 14 shows boxplots of these MDS results for each group. The SPSS Explore
procedure identified three outliers (points labeled 10, 18, and 34), and these were excluded from further analyses.

Figure 14. MDS mean X-axis coordinates for each of the five groups of mice.

Figure 15 shows the means of the five groups with the outliers removed, demonstrating how groups were pooled for final analysis. The two groups of controls (C57BL/6J & EphA4^{+/+}) are displayed on the left. The two groups of mutants (EphA4^{+-} and EphA4^{-/-}) are displayed in the middle. The BALB/c mice are displayed on the far right. One-way Analysis of Variance of the MDS result from these three groups shows a highly significant difference ($F_{2,54}=14.5, p<.001$), with the LSD post-hoc test showing that each group was significantly different from every other group ($p<.033$; and that difference was between the BALB/c and the combined groups of C57BL/6J). Of possibly greater interest is the significant difference between the C57BL/6J and mutant mice
(F_{1,29}=6.6, p=.015). Figure 16 illustrates this finding. MDS finds a significant difference between mutant and non-mutant mice, in that within-group differences (distances) are less than between-group differences (distances).

Figure 15. Clustered groups of mice from MDS result with outliers removed.
Figure 16. MDS mean X-axis coordinates for control and mutant mice.
Discussion

The acoustic startle response (ASR) has been used to study afferent processing. However, efferent influences on the ASR should be carefully considered. The presumed stereotypical motor response appears not to be stereotypical after all. It may not be surprising to observe differences between the startle responses of BALB/c and C57BL/6J mice because these are inbred strains with many other differences. Yet, within the C57BL/6J background, EphA4 mutations, both heterozygous and homozygous, had a significant effect on the detailed pattern of the unconditioned ASR. Thus, EphA4 mutation appears to affect efferent pattern generation, and the ASR in mice appears to be useful for studying this efferent impact on motor responses.
References


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