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Effects of Eph/ephrin mutations on pre pulse inhibition in mice

Andrea Marie Liuzzo
James Madison University

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Andrea Marie Liuzzo

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Introduction to the Reader

The first part of this dissertation is in manuscript format and includes the introduction, materials and methods, results, discussion and conclusion in preparation for publication. The second part of this dissertation is an expanded literature review meant to provide the reader with further literary background and insight towards this project and begins after the conclusion of the manuscript. Please refer to the Table of Contents for specific page numbers.

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Abstract

The acoustic startle response (ASR) is a reliable reflexive behavioral response in mammals elicited by an unexpected intense acoustic startle eliciting stimulus (SES). It is mediated by a sub-cortical pathway that includes the inferior colliculus (IC). The ASR amplitude can be measured with an accelerometer beneath the subject attached to the cage, and can be decreased in amplitude by presenting a less intense, non-startling stimulus 20-300 ms before the SES. This reflexive decrement in ASR is called pre pulse inhibition (PPI) and indicates that the relatively soft pre pulse was heard. Murine species have been used to study this response for psychoacoustical estimates of hearing thresholds and to understand the effects of genetic mutations on the ASR, PPI and the afferent auditory neural pathway. The Eph/ephrin signaling pathway is known to be important in directing developing auditory afferents, including connections to various subdivisions of the IC. In this experiment, we measured the effect of Eph/ephrin mutations on PPI in mice with a control strain (C57BL/6J), a strain with compromised EphA4 signaling ($EphA4^{lacZ}$), and a knockout ephrin-B3 strain ($ephrin\text{-}B3^{null}$). The control strain and $EphA4^{lacZ}$ strain showed robust PPI (up to 75% decrement in ASR) to an offset of a 70dB SPLrms background noise at 50ms before the SES. Ephrin-B3 knockout mice were only marginally significant in PPI (< 25% decrement) to the same conditions. This reduction in PPI highlights the significance of ephrin-B3 in the developing afferent auditory system by ways of auditory behavioral measurement. Thus, different mutations for certain members of the Eph/ephrin signaling family produce a full range of changes in PPI, from minimal to nearly maximal. This technique can be easily adapted to study other aspects of hearing in a wider range of mutations. Along with

ongoing neuroanatomical studies, this allows careful quantification of how the auditory anatomical, physiological and now behavioral phenotype is affected by changes in the Eph/ephrin genotype.

Part I: Manuscript Introduction

Acoustic Startle Reflex and Pre pulse Inhibition

The acoustic startle response (ASR) is a motor response in mammals elicited by and directly following the presentation of an unexpected intense acoustic stimulus. Behaviorally, it is a rapid contraction of skeletal muscles that is considered a defensive response (Swerdlow et al., 2000). This behavioral response can be measured with the use of an accelerometer placed directly beneath the subject (Allen & Ison, 2010). The magnitude of this response can be altered by a variety of factors including the addition of a non-startling stimulus (pre pulse) presented before the startle eliciting stimulus (SES) (Gaese et al., 2009). Reflex Modification Audiometry (RMA) is a rapid and efficient method of utilizing the ASR and its attenuation in amplitude by the addition a pre pulse stimulus to estimate behavioral thresholds (Allen & Ison, 2010; Ison et al., 1998).

Pre pulse inhibition (PPI) is a form of RMA where a non-SES stimulus is presented 1-300 ms before the SES; the perception of this pre pulse stimulus will then reduce ASR amplitude. The PPI paradigm has been utilized in various research efforts as it is sensitive to manipulations in many parameters, is reliable across time, is easily quantified and is controlled by a simple neural circuit that is present across mammalian species (Swerdlow et al., 2000). It has advantages over operant conditioning paradigms in that it does not require training or reinforcement efforts (Fitch et al., 2008).

Reliability of the mouse model

Mice are useful to better understand the genetic bases of the mammalian auditory system and how genotype can affect auditory development. There is remarkable structural similarity between the human and murine auditory system; the mouse genome shows 80%

homology with the human genome (Kikkawa et al., 2012) which makes the murine species a reliable mammalian model to better understand factors that influence the development and functioning of the system. This led to the utilization of RMA in rodents to better understand the effects of genetic mutations on the mammalian auditory nervous system (Allen & Ison, 2010; Ison et al., 1998).

The neural circuit mediating the ASR in the murine species is strikingly similar to humans, making this response reliable for cross-species, translational research (Swerdlow et al., 2000). Stimuli enter the cochlea, the signal is then transmitted to the auditory nerve, to the cochlear nucleus (CN), to the nuclei of the lateral lemniscus (NLL), to the nucleus reticularis pontis caudalis (PnC) located at the head of the reticulospinal tract and to the spinal motor neurons which then innervate flexor and extensor muscles of the body (Ison et al., 1998; Fitch et al., 2008). The addition of the pre pulse stimulus inhibits the ASR by interfering with the neural circuit at the level of the inferior colliculus (IC) where excitatory input is sent to the pedunculopontine tegmental nucleus which inhibits the PnC of the ASR neural pathway (Fitch et al., 2008).

Allen & Ison (2010) studied the effects of inter-stimulus intervals (ISIs) of the pre pulse stimulus in CBA/CaJ inbred mice via an offset paradigm, an onset paradigm and a speaker swap of 180 degrees azimuth. The most robust PPI was elicited by the offset of a 70 dB broadband noise (filtered from 1k to 50k Hz) with an ISI of 50ms. The present study repeats this paradigm, seeking to obtain the same results in wild-type mice and showing a range of responses in mutant mice.

Eph/ephrin signaling

The Eph receptors and their ligands, the ephrins, play a strong role in the development of the auditory system in mice by patterning the tonotopic structure from the auditory periphery to the brainstem, and up to the auditory cortex (Cramer, 2005; Intskirveli et al., 2011). Eph/ephrin signaling pairs involve interactions between two families; As and Bs. The mouse organ of Corti and spiral ganglion cells have strong expression of EphA4, EphB3, ephrin-B2 and ephrin-B3 (Pickles, 2003). Studies in EphB2 knockout mice have proven that the EphB2 protein is important in the development of the peripheral vestibular system with these mice exhibiting circling behaviors (Cramer, 2005; Cowan et al., 2000; Pickles, 2003). Current research by Gabriele et al. (2011) and Wallace et al. (2013) has illustrated the importance of Eph/ephrin signaling in the mammalian IC prior to the onset of hearing. The use of DiI-labeling showed the involvement of the EphA4 and ephrin-B2 protein signaling in the development of the tonotopic organization of the mouse IC (Gabriele et al., 2011). Mice lacking a portion of the ephrin-B2 protein did not display a reliable topographic pattern of lateral superior olive (LSO) to IC projections when compared to control strain (Wallace et al., 2013). Therefore, it is our thought that mutant Eph/ephrin mice will display altered PPI when compared to a control strain.

General Statement of the Problem

Currently, there is a lack of literature using Pre Pulse Inhibition (PPI) to understand the effects of Eph/ephrin proteins on the development of the mouse auditory system. Therefore, the aim of this experiment is to better understand the behavioral effects of Eph/ephrin protein signaling by comparing Eph/ephrin mutant mice to a control strain

using Allen & Ison (2010)'s PPI procedures. The following is a behavioral evaluation of mutations that have been studied genetically, histologically, and physiologically.

Materials and Methods

Subjects

Mice (n= 17) of two different Eph/ephrin mutations and a control group were used. The control group consisted of seven C57BL/6J mice, the background for the EphA4^{lacZ} mutations, plus two wild-type offspring of heterozygous EphA4^{lacZ} parents that lacked the mutant allele. Two strains of mutant mice were tested; ephrin-B3^{null} (n=4, two homozygous, 2 of heterozygous) and EphA4^{lacz} (n=4, all homozygous). Mice varied between the ages of 31 days and 75 days and were tested twice. The average age at the first test was 37 (+/- 8.9) days. The average between the first and second test was 15.47 (+/- 5) days. All mice were tested before the expected onset of age-related hearing loss of 8 months in the C57BL/6J strain (Ehret, 1976). All mice were group-housed (4-6 mice per cage) in a BioZone MiniSmart Rack System in a controlled constant climate. All testing was done during the daylight hours. Food and water were always available except during testing which lasted approximately 60 minutes. The James Madison University Institutional Animal Care and Use Committee (IACUC) approved all procedures prior to experimentation.

Apparatus & Stimuli

Mice were tested in a 5cm inside-diameter by 12.5 cm long San Diego instruments Plexiglas tube attached to an accelerometer taken from the SR-LAB mouse-testing chamber. This tube was placed in the middle of a 7’ x 7’ (2.13 meters x 2.13 meters) Industrial Acoustic doubled walled, double floored sound attenuating booth. The chamber was 18” (45.7 cm) beneath a Ross Audio Systems TW 30 compression tweeter. The pre pulse stimulus was presented via a Tucker Davis Technology ES1 compression

tweeter 15cm to one side of the testing chamber. Startle eliciting stimuli (SES) were 110dB SPLrms, 15ms broad-band noise, high pass filtered at 8k Hz, linear gated with a 10 microsencond rise/fall time. Calibration showed significant energy up to 50k Hz, 110dB SPLrms in the 768 Hz to 50k Hz band. The SES noise was generated using a Tucker Davis Technology RP2 Real-Time Processor amplified by a Crown XLS202 amplifier. The pre pulse stimulus was an offset of the continuous background noise high pass filtered at 4k Hz (1k Hz to 100k Hz bandwidth, 70dB SPLrms +/- 1dB SPLrms), therefore the background noise and startle eliciting stimulus (SES) were very similar; the pre pulse cue being the offset of the background noise. The offset was linear gated. The force of the startle reflex was transduced by an accelerometer attached to the bottom of the testing tube. The voltage from the accelerometer was low-pass filtered at 1k Hz and amplified times 100 (20 dB + 20 dB) by a Krohn-Hite model 3343 filter and input to a TDT-RP2.1. This input was digitized at 200k Hz for 100ms starting at the same time that the startling stimulus began. Test trials began two minutes after the mouse was placed in the testing chamber (two minute acclimation period), and testing continued for about 60 minutes.

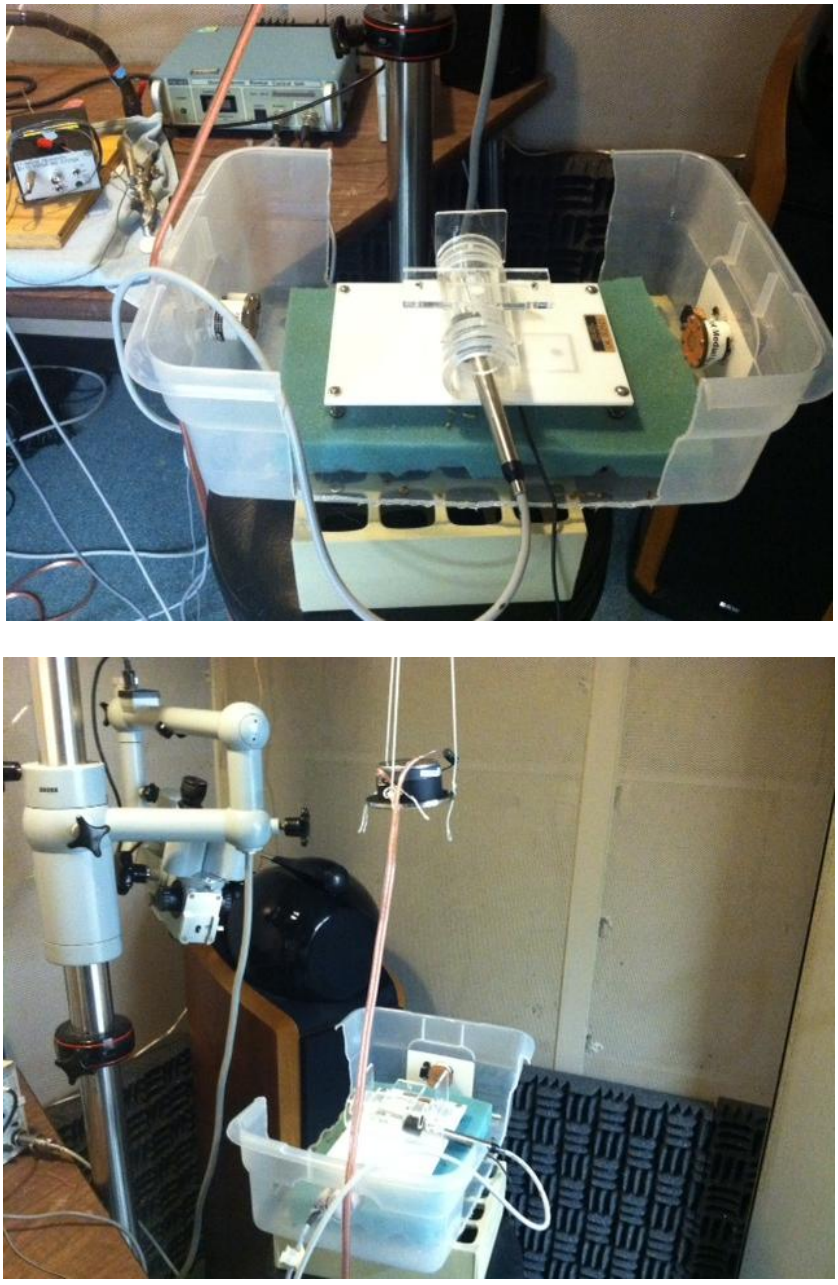

Figure 1: A photo of the test chamber and testing set-up

General Procedures

The pre pulse in this experiment was an offset of the 70dB SPLrms stimulus at 90 degrees azimuth to the mouse. Sixteen different conditions were repeated in 11 different blocks. There were 13 different interstimulus intervals (ISI: time between offset of carrier

stimulus and presentation of eliciting stimulus). The 13 different ISI conditions were 1, 2, 5, 10, 30, 40, 50, 60, 100, 150, 200 and 300ms. Each block contained these 13 ISIs plus two no pre pulse baseline control trials and a No-SES control trial to measure background activity, for a total of 16 trials/blocks. The intertrial interval randomly varied between 15 and 25 seconds. These 16 different trials were presented in 11 different blocks, with the order of trials randomized within each block. RMS voltage (100ms from start of the startle stimulus) was calculated for each trial. Pre pulse inhibition (PPI) scores were calculated as a ratio of the subject's mean response amplitude in the pre pulse stimulus condition (ASRp) compared to the control baseline measure with only eliciting (startle) stimulus and no pre pulse (ASRc) using the formula: PPI = 1 – [ASRp/ASRc] as in Allen & Ison, 2010.

The methods above replicated Allen and Ison (2010), except that our SES was 10dB less intense. In addition, four "mock subjects" were run exactly as the mice, except there was no mouse in the chamber. All subjects were run with the lights off. Calibrations were done with an Agilent 35670A Spectrum Analyzer, ¼" microphone (Bruel & Kjaer 4939) placed in the center of the Plexiglas tube, amplified by a Listen, Inc. Sound Connect amplifier.

Results

There was a highly significant effect of Eph/ephrin mutations: repeated-measures ANOVA in Pre Pulse Inhibition (PPI) with 26 within-subjects measures (13 ISIs from 1 to 300 ms at two testing times) from each of 17 mice in three groups (9 wild-type and 4 each ephrin-B3^{null} and EphA4^{lacZ} mutations) showed a large between-subject effect ($F_{2,14}$=36, p<.001, η^2 = 84%). Post-hoc pairwise (LSD) comparisons showed the wild-type and EphA4^{lacZ} groups to be similar (p=.64) and each different from the ephrin-B3^{null} (p<.001). Among the within-subjects effects there is a strong effect of ISI (Wilk's λ=.02, p=.03), with the strongest planned polynomial contrast being the quadratic ($F_{1,14}$=187, p<.001, η^2=.93, indicating the expected curved function of ISI as seen in Fig. 1. There was no effect of time (Wilk's λ=.9, p=.24) nor any interaction with time (p>=.1); that is, no effect of the repeated test after an average of 15 days.

Because there was no effect of the repeated testing (time), the PPIs at each ISI were averaged over the two tests for each mouse. Data from four 'no mouse' (empty chamber) controls tests were added for a repeated measures ANOVA with one within-subject factor (13 ISIs) and one between-subject factor (4 groups: C57BL/6J [WT], EphA4^{lacZ}, ephrin-B3^{null}, and empty-chamber). These means are shown in Fig. 1. Post-hoc tests showed the ephring-B3^{null} to be different than the no-mouse controls (p=.007), indicating that the least responsive group of mice still showed significant PPI; the ephrin-B3^{null} mutations heard something.

Half of the ephrin-B3^{null} mice were homozygous and half heterozygous mutants. There was no difference between these genotypes ($F_{1,2}$=.01; p=.93; from a repeated

measures ANOVA of only the 4 ephrin-B3^{null} mutant mice; also showing no effect of time or ISI).

Looking now at only the trials where there was no pre pulse (startle alone, labeled ASRc in Allen and Ison, 2010, and no-sound controls in the three groups of mice), repeated measures ANOVA showed no main effects of group or time, but there was a possible group-by-time interaction, p=.03 to .13 depending on the approach to analyzing within-subject factors. For univariate group-by-time tests: $F_{2,14}$=4.359, p=.034, $_p\eta^2$=.384 for baseline; $F_{2,14}$=2.422, p=.125, $_p\eta^2$=.257 for startle alone. For the group-by-time interaction from 2 (SES or none) x 2 (times) multivariate tests: Pillai's Trace = .518, $F_{4,28}$=2.445, p=.070, $_p\eta^2$=.259; Wilk's Lambda = .494, $F_{4,26}$=2.752, p=.049. $_p\eta^2$=.297; Hotelling's Trace = 1.0003, $F_{4,24}$=3.010, p=.038, $_p\eta^2$=.334). The ephrin-B3^{null} mice startled less on the 2nd test: t_3=4.043, p=.027 two-tailed, Cohen's d > 2 (.574/.284), in a paired-sample t-test comparing ASRc, the response to the SES alone in test 1 with that in test 2. The recorded startle response (mean $\pm$ s RMS voltage of 7.7$\pm$1.9 mV was about half on the 2nd test compared to the 1st test in this group only (13.4 $\pm$ 4.5 mV). There was no significant decrement on baseline trials (t_3=2.195, p=.116, two-tailed) with RMS voltages of 2.6$\pm$1.6 mV on the first test and 1.4$\pm$ 0.6 mVon the 2nd test.

There was no difference between the groups in their age at testing ($F_{2,16}$=3, p=0.09). There was no difference between the groups in this intertest interval ($F_{2,16}$=0.4, p=0.66).

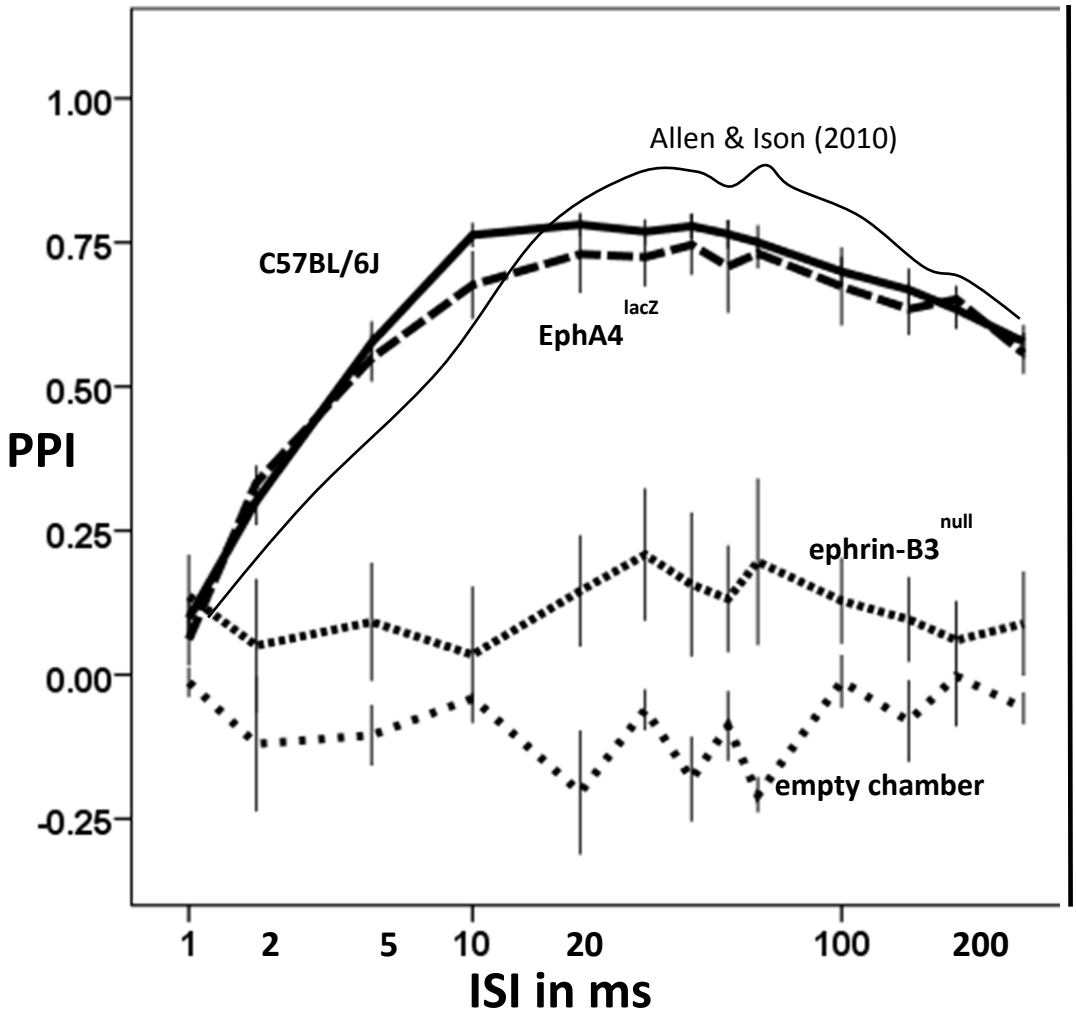

Fig. 2. Pre pulse inhibition of startle amplitude elicited via offset of the ongoing 70dB SPLrms background noise in varying ISIs before the startle eliciting stimulus (110dB SPLrms). Results of amount of PPI relative to ISI of the control strain (C57BL/6J), EphA4^{lacZ} mutant strain, and ephrin-B3^{null} strain compared to the results of Ison & Allen (2010) and averaged data from a chamber with no mouse (empty chamber). Averaged responses from the EphA4^{acZ} strain is near-normal while averaged ephrin-B3 ^{null} responses were only marginally significant over the empty chamber condition. Error bars represent one standard error.

Discussion

The goal of this study was to study the effects of various Eph/ephrin mutations on hearing as revealed through the acoustic startle reflex (ASR) and Pre Pulse Inhibition (PPI). We repeated the procedures of Allen & Ison (2010) and extended the results to different Eph/ephrin mutations. To our knowledge, these are the first behavioral data on the effects of Eph/ephrin signaling on auditory responsiveness in mammals. PPI reveals the full range of effects of Eph/ephrins on hearing from near minimal to near maximal effects. The results of this experiment prove that the signaling of the Eph/ephrin proteins can be important for behaviorally mediated responses to sound stimuli. We found the response to be quite stable over time.

Data from our wild-type mice closely replicated the results of Allen and Ison (2010). The slight decrement in the responsiveness of our mice could be due to our 110dB SPL SES compared to the 120dB SPL SES of Allen and Ison.

Pre pulse inhibition

Inhibition of the ASR elicited by a pre pulse cue of background noise offset was a strong response in the control strain. The control group (C57BL/6J and wild-type) produced the most robust PPI response with a peak PPI of 0.778 (78% reduction of ASR) at 40ms of ISI. The EphA4^{lacZ} heterozygous mutant mice showed near-normal behavioral responses when compared to the control strain with a peak PPI response of 0.746 (75% reduction of ASR) at about 40ms ISI. Both the control strain and the EphA4^{lacZ} strain displayed increasing PPI with increasing ISI until about 40ms where the response saturated. These results are consistent with those of Allen & Ison (2010) where an ISI of 50ms produced the greatest PPI for an offset paradigm with a saturation and subsequent

decrease of the PPI response after 50ms. The ephrin-B3^{null} strain was only marginally significant in its responses, with PPI measures of less than 21%; but still significantly above the PPI values in the empty-chamber condition. There was no evidence of a correlation between PPI and ISI in the ephrin-B3^{null} strain.

Effects of Eph/ephrin signaling

It is thought that various alterations in Eph/ephrin expression will produce varying behavioral responses. This experiment has illustrated that the heterozygous EphA4^{lacZ} strain, with only a partial alteration in the EphA4 protein rendering it incapable of reverse signaling (Eph to ephrin), do not show behavioral differences when compared to the control group strain. This leads us to believe that a small alteration in the EphA4 protein may not affect behavioral auditory responses mediated by the Pre Pulse Inhibition (PPI) neural circuit. Meanwhile, a complete knockout of the ephrin-B3 protein significantly changes the behavioral results of the PPI paradigm, illustrating the importance of ephrin-B3 in the PPI neural circuit. The results of this experiment show that different aspects of Eph/ephrin signaling may play different roles in the development of the afferent auditory system and have a direct effect on the mediation of the PPI paradigm. Ephrin-B3, specifically, may play an important role in mediating PPI.

In addition to an alteration of the PPI neural circuit, the diminished responsiveness of the ephrin-B3^{null} strain results may also be caused by increased hearing thresholds. Increased PPI, or a reduction in ASR amplitude, is directly related to the perception of pre pulse stimulus intensity. That is, a more intense pre pulse stimulus will produce a greater reduction in ASR amplitude (Allen and Ison, 2010). Shearer et al. (2012) studied the effect of Eph/ephrin mutations in mice on tone-burst and click evoked

ABRs and found that the ephrin-B3^{null} strain had the highest ABR thresholds in all conditions when compared to the EphA4^{lacZ} and the C57BL/6J control strain. With increased hearing thresholds, the ephrin-B3 mice may have had a reduced sensation level of the background stimulus, therefore perceiving the pre pulse stimulus at a softer level; this, in turn, reducing PPI. Therefore, the ephrin-B3 protein may be important for overall afferent auditory development, and not just the PPI neural circuit. Meanwhile, the EphA4 strain demonstrated only slightly increased ABR thresholds when compared to the control strain indicating a lesser effect of the Eph/ephrin mutation on hearing. Our experiment correlated well with Shearer (2012)'s results, as our EphA4 strain demonstrated only a slight reduction in PPI when compared to controls.

Importance of further research

To further delve into the importance of Eph/ephrin proteins, researchers may want to alter the pre pulse stimulus and include other mutations to better understand how Eph/ephrin signaling alters the afferent auditory system. This sets up ideas for further experimentation utilizing the Pre Pulse Inhibition (PPI) paradigm in various Eph/ephrin mouse strains. Various psychoacoustical parameters of the pre pulse could be varied in future studies to test thresholds to onsets, details of the psychometric function, receiver operating characteristics, localization, and gap detection.

Conclusions

Eph/ephrin proteins are important in both peripheral and central auditory development in the mammal. Although neuroanatomical research efforts have mapped where these proteins are expressed, there has been little research on the behavioral effects of Eph/ephrin mutations. This experiment lays the groundwork for a better understanding of how Eph/ephrin signaling may alter auditory behaviors. In summary, EphA4^{lacZ} mutant mice have near-normal PPI responses to broadband noise offsets. Meanwhile, the ephrin-B3^{null} strain without the ephrin-B3 protein, shows highly altered PPI responses with very little reduction in ASR amplitude to noise offset. It appears that much can be learned about the ultimate, functional, behavioral effects of Eph/ephrin mutations through PPI in mice.

Part II: Expanded Literature Review

1. Introduction

Recently, researchers have begun to better understand the importance of Eph/ephrin signaling in the development of both the peripheral and central auditory nervous system. It is now well understood that the Eph receptors and their ligands, the ephrins, are highly expressed in the auditory periphery of the mouse, including the cochlea and spiral ganglion (Pickles, 2003). Emerging evidence has highlighted the expression of Eph/ephrins in the central auditory nervous system (Gabriele et al., 2011) and has begun to illustrate the importance of Eph/ephrin expression in topographic patterning of auditory afferent neural pathways (Wallace et al., 2013). Shearer (2012) studied the effects of different mutations of Eph/ephrin genetics on mouse hearing via Auditory Brainstem Responses (ABRs) and found that ephrin-B3 knockouts, or mice lacking an entire gene coding for the ephrin-B3 ligand, had the greatest thresholds to ABR stimuli (i.e. worse hearing). Although electrophysiological measures provide reliable data on brainstem responses to sound, the gold standard of hearing perception is through behavioral measures. Therefore, the goal of the current study was to obtain reliable auditory behavioral responses from the various strains of Eph/ephrin mice and compare these results to normal controls. We aimed to repeat the methods of Allen & Ison (2010) using pre pulse inhibition (PPI) to the acoustic startle reflex (ASR) to determine the differences in hearing perception between our three strains of mice.

2. The Murine Model

The murine species is a reliable mammalian model used to better understand the effects of genetic mutations. The mouse model is an important scientific tool to better understand the underlying bases of the mammalian auditory system and how neural and

biological changes within the species can affect auditory development. Radziwon et al., (2009) state that "a large number and variety of genetically engineered mouse strains make mice good models for studies that seek to identify genetic factors likely to contribute to various forms of human deafness," pg. 961. There is remarkable structural similarity between the human and murine auditory system that increases the validity and importance of the research being conducted in mice. In fact, the "mouse genome shares 80% homology with the human genome" (Kikkawa et al., 2012, pg. 86). This similarity in genetics leads to the fact that mouse genes involved in hearing have strong genetic sequence similarities and functions to human genes (Kikkawa et al., 2012). Therefore, we feel as though this study will provide useful information on the effect of Eph/ephrin signaling on the mammalian auditory system that can contribute to the better understanding of the importance of these proteins on the mammalian auditory system.

3. Murine Auditory System

The mouse auditory system is functional by 10 days post natal (Mikaelin & Ruben, 1965). That is, the external ear and middle ear, cochlea and auditory pathways are fully formed. The external and middle ear, specifically, are closed at day 8 post natal, while the mouse cochlea, comprised of two turns, basal and apical, is not fully developed until day 10 post natal when the basilar membrane has an adult-like appearance. Meanwhile, behavioral and electrophysiological studies have shown that the central auditory nervous system of the mouse is not fully developed until day 10 post natal (Mikaelin & Ruben, 1965) where the Preyer reflex, a behavioral response of the central auditory nervous system, and the N1 and N2 responses, electrophysiological measures of sound-evoked activity in the VIIIth nerve and spiral ganglion cells, were not able to be

measured in mice until the 10th day. The mouse auditory system has limits from 500 Hz to 100 kHz (Ehret, 1976), and is most sensitive between 8k and 24k Hz (Radziwon et al., 2009); the most sensitive frequency being 15k Hz (Ehret, 1976). Therefore, it was imperative that all auditory stimuli were well within the hearing limits of the mouse throughout this experiment. Similar to all mammalian species, the central auditory nervous system of the mouse is tonotopically organized, where topographic projections begin in the periphery and continue through the midbrain to the primary auditory cortex. The tonotopicity of the auditory system is preserved throughout the brainstem, where each axonal fiber has a best frequency of which it is most sensitive to. This tonotopicity creates frequency maps in the nuclei of the auditory brainstem. The inferior colliculus (IC), the nucleus of the auditory system located in the midbrain, is highly organized based on frequency. More specifically, the central nucleus of the IC represents high frequencies in the ventromedial portion and represents low frequencies in the dorsolateral portion. The control strain used in this study (C57BL/6J) shows severe deterioration in hearing beginning at 8 months of age (Ehret, 1976); therefore it was imperative that no mouse in this study was tested at 8 months or older.

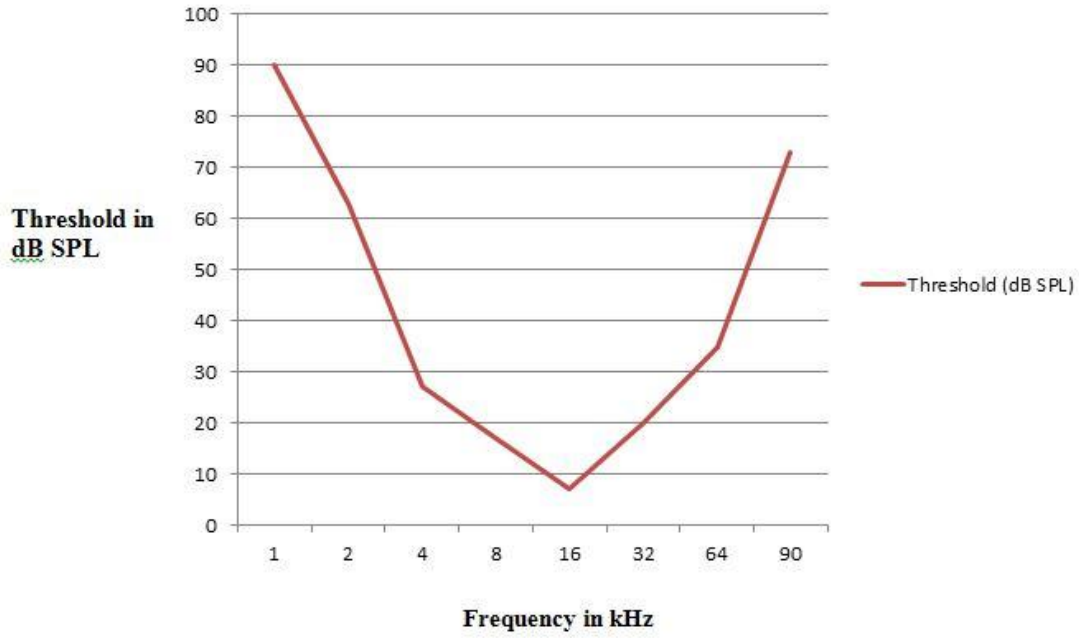

Figure 3: an audiogram of the mouse mapping the frequencies to which the species is most sensitive to (adapted from Heffner & Heffner, 2007).

4. Acoustic Startle Reflex & Pre Pulse Inhibition in Mammals

4.1 Introduction to Pre Pulse Inhibition (PPI) and its effect on the Acoustic Startle Reflex

The acoustic startle reflex is a motor response directly following the presentation of an unexpected intense stimulus (i.e. a loud noise), (Fitch et al., 2008); considered to be a defensive response (Swerdlow et al., 2000). Pre pulse inhibition, specifically, is a behavioral measure that is efficient in determining whether an animal has detected a signal (Fitch et al., 2008). This decrease of the startle reflex is thought to be a behavioral representation of sensorimotor gating, or the selective transmission of sensory information to the motor system (Burgess & Granato, 2007). Here, the behavioral

regulation of motor responses aims to filter out superfluous sensory information and only relay important sensory stimuli to the motor system to create a behavioral reaction (Burgess & Granato, 2007). In PPI, sensorimotor gating is evident as the "degree to which the initial weak *sensory* event (pre pulse) inhibits the reflexive *motor* response to the subsequent intense sensory event (startling stimulus)" (Swerdlow et al., 2000 pg. 186). The pre pulse stimulus is a non-startling stimulus presented 20-500ms before the startle eliciting stimulus that will reduce the amplitude of the acoustic startle reflex (ASR) if it is perceived, (Fitch et al., 2008; Allen & Ison, 2010; Zavitasanou et al., 1999). The amount of ASR amplitude reduction is directly correlated to the ability to detect the pre pulse stimulus; that is, if the pre pulse stimulus is barely detected the ASR amplitude will only reduce slightly, whereas if the pre pulse stimulus is easily detected the ASR amplitude will be greatly reduced (Fitch et al., 2008). In the present study, we chose an offset of the ongoing background noise as the pre pulse stimulus presented from 1ms to 300ms before the startle eliciting stimulus (ES).

4.2 ASR & PPI Neural Circuit in the Mouse

Mouse models are highly advantageous when studying pre pulse inhibition in relation to the ASR as the brain circuitry involved in this response poses, "striking similarities across species from rodents to humans, making startle plasticity ideal for cross-species translational research" (Swerdlow et al., 2000, pg. 186). Developmentally, the acoustic startle reflex increases in robustness throughout the first month of life in the C57BL/6J mouse. This development is parallel with the developmental of auditory function as measured by electrophysiogical findings (compound action potentials of the VIIIth nerve) (Ison et al., 1998). As the C57BL mouse ages, the reflex deteriorates

beginning at middle age (6-10 months) (Ison et al., 1998). Therefore, measures were taken to ensure that testing was done before the onset of middle age.

The neural circuit mediating the ASR begins at the cochlea; the signal is then transmitted to the auditory nerve, to the cochlear nucleus (CN), to the nuclei of the lateral lemniscus (LL), to the nucleus reticularis pontis caudalis (PnC) at the head of the reticulospinal tract, to the spinal motor neurons which then innervate flexor and extensor muscles of the body (Fitch et al., 2008; Ison et al., 1998). Electromyography measures of the ASR latency are measured at 10 – 20 milliseconds in rodents (Ison et al., 1998). The addition of a pre pulse stimulus then inhibits the ASR by interfering with the pathway. Specifically, the pre pulse stimulus travels from the cochlea, to the CN, to the nuclei of the LL, to the inferior colliculus (IC). Once at the level of the IC, an excitatory input is sent to the pedunculopontine tegmental nucleus which, then, inhibits the PnC (the major nucleus involved in the ASR), (Fitch et al., 2008). The prepulse stimulus inhibits the ASR by inhibiting the activity of the PnC. There is some thought that structures higher in the central auditory nervous system (CANS), such as the medial geniculate body, are additionally involved in the pre pulse inhibition phenomenon, but this understanding is not yet complete (Fitch et al., 2008). In summary, the pre pulse inhibitory effect reflects the, "activation of 'hardwired' centrally mediated behavioural gating processes." (Swerdlow et al., 2000).

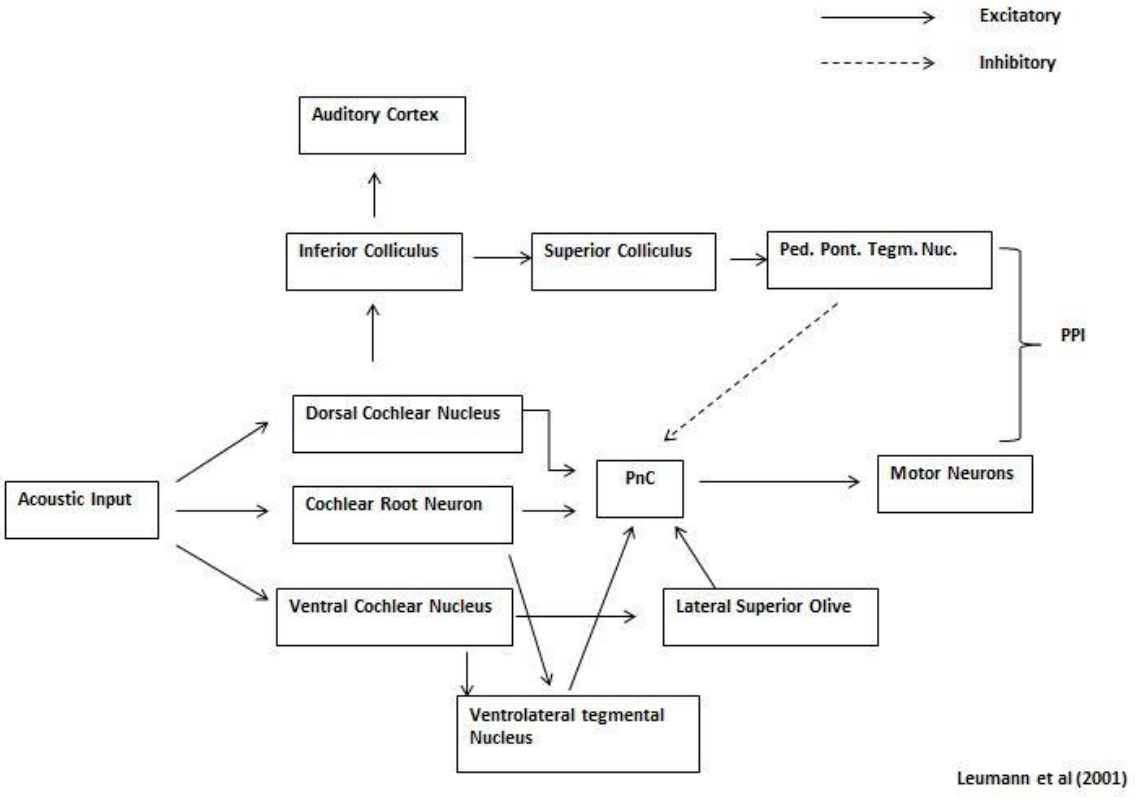

Figure 4: A representation of the neural circuit mediating both the ASR and PPI in mammals adapted from Leumann et al. (2001).

4.3 Pre Pulse Inhibition as a Behavioral Paradigm

The paradigm that was used in this study is known as Reflex Modification Audiometry (RMA) - a method that is both rapid and efficient at determining whether or not an animal has detected a signal by observing its reaction to a reflex-inducing stimulus (Ison et al., 1998, Allen & Ison, 2010). In this case, we used the pre pulse inhibition (PPI) paradigm, where, as discussed before, the pre pulse (an offset in the background noise) reduces the acoustic startle reflex as measured by the change in voltage RMS amplitude from an accelerometer beneath the mouse. Although we did not specifically find auditory thresholds in this experiment, many researchers utilized reflex modification audiometry

and pre pulse inhibition (PPI), specifically, to find behavioral auditory thresholds in rodents; therefore, pre pulse inhibition is a type of RMA (Allen & Ison, 2010). Swerdlow et al. (2000) described the advantages of the PPI paradigm stating: "it is easily quantified, it is sensitive to a variety of parameters that are easily manipulated and can yield both increases and decreases in response, it is controlled by a simple neural circuit, it is measurable across species, it exhibits predictable forms of plasticity and it is sensitive to drug effects," pg. 196. Previous research has indicated that RMA, and the pre pulse inhibition paradigm specifically, is a sensitive and objective test to measure behavioral responses within a short time period of days or weeks (Ison et al., 2010; Fitch et al., 2008).

Reflex Modification Audiometry (RMA), specifically Pre Pulse Inhibition (PPI), has several advantages over the well-utilized operant conditioning paradigm in animal research. Operant conditioning is a useful technique in animal research to study behavioral responses of animals, whereby the animal is rewarded when the desired behavior is performed. Unfortunately, operant conditioning paradigms may take longer periods of time in order to train the animal for the desired task (Fitch et al., 2008). Additionally, operant conditioning paradigms utilize water or food deprivation in order to reward the animal. RMA, including the pre pulse inhibition (PPI) paradigm, does not require training or reinforcement in order to obtain the desired behavior, as it is based on a behavioral reflex (Ison et al., 1998; Allen et al., 2008; Fitch et al., 2008) and still maintains a sensitive measure for behavioral testing. Pre pulse inhibition, a form of sensorimotor gating (or sensorimotor inhibition), is not a form of conditioning as the response occurs to the first exposure of the pre pulse cue and, therefore, requires no

training, (Swerdlow et al., 2000). Additionally, this response does "not exhibit habituation or extinction over multiple trials," (Swerdlow et al., 2000). Hence, RMA can be useful in obtaining the desired behavioral response quickly without disrupting the well-being of the animal.

An offset paradigm, as used in this current study and also employed by Allen & Ison (2010), tends to elicit very robust Pre Pulse Inhibition (PPI). One theory for the use of an offset paradigm is that the ongoing background noise may facilitate the Acoustic Startle Reflex (ASR), where the ASR is highest in amplitude when there is an ongoing background noise present. Any offset of this background noise, then, will be a pre pulse cue and subsequently reduce the ASR amplitude (Ison et al., 1998). Additionally, it is thought that the background noise will cause a behavioral habituation pattern, where the subject will become used to the ongoing background noise. Therefore, an offset of the background noise will inherently cue the animal for the startle eliciting stimulus (SES) and reduce ASR amplitude (Ison et al., 1998). The final theory, and one that Ison and colleagues have adopted as the most likely theory, is that the inhibition of the ASR due to the noise offset may be a result of a form of forward masking. In this theory, ASR inhibition is a form of behavioral gap detection dependent on inhibitory circuits of the lower brainstem (Ison et al., 1998). Additionally, the offset pre pulse utilized in this experiment is dependent on the gap-detection abilities of the mouse. More specifically, research has indicated that the inferior colliculus (IC) is highly involved in the detection of gaps in noise within the mouse auditory system (Walton et al., 1997). One experiment researched the neural responses to silent gaps within white-noise carriers within specific neurons of the CBA mouse IC and then compared these results to behavioral estimated of

gap detection. They discovered that the majority of the neurons within the mouse IC were responsible for the temporal discharge pattern necessary to behaviorally detect gaps in noise (Walton et al., 1997). Therefore, the use of the offset paradigm within this experiment may be useful to determine the integrity of the gap-detection neurons in the IC of the wild-type and mutant mice, a structure that is known to be affected due directly to Eph/ephrin mutations in mice (Cramer, 2005).

5. Effects of Genetic Mutations on PPI

It is well known that much of the brain-circuitry regulating the PPI effect is sensitive to the genotype (Swerdlow et al., 2000); that is, changes in genetic make-up may, in fact, affect the pathway for the PPI effect. It is for this reason that the PPI paradigm has been utilized, and was utilized in this experiment, to better understand the effects of genetic mutations on the central auditory system, specifically the neural circuitry mediating the ASR and PPI. Two major subsets of genetic effects have been studied using the PPI paradigm in mice: inbred strain studies and genetically mutated mice studies (Geyer et al., 2002). Genetically mutated mice, as used in this current experiment, have been able to target and alter specific proteins that are thought to regulate the unconditioned PPI response. For example, serotonin receptor deficient mice have been studied via PPI to test the hypothesis that serotonergic receptor-mediated processes regulate sensorimotor gating (Geyer et al., 2002). Dulawa et al. in the late 1990s discovered that mutant mice lacking the serotonin-1B receptor have increased PPI levels. This evidence, then, suggests that the serotonin-1B receptor is highly important in the processes underlying the PPI response (Geyer et al., 2002).

Additionally, PPI has been used to measure changes in nervous system in subjects with other altered neurotransmitters or enzymes. Genetic studies have also looked at the effect of altered dopamine receptors in mice, where dopamine has been shown to regulate sensorimotor gating (Geyer et al., 2002). The nicotinic acetylcholine receptor (nAChR) has been postulated to be important in all sensorimotor gating including PPI (Geyer et al., 2002). Alpha-bungarotoxin (alpha-BTX) binds with high affinity to the alpha7 nAChR; injections of alpha-BTX have been shown to disrupt hippocampal sensory gating (Geyer et al., 2002). Van de Buuse et al. (2003) looked at the effect of aromatase enzyme knockout; specifically, these mice lacked a functional aromatase enzyme and were unable to convert testosterone into estrogen. Male aromatase knockout mice displayed an age-related reduction of PPI proving that this neural pathway depends on dopaminergic activity mediated by estrogen within the neural pathway. Based on this evidence, it is clear that PPI, and sensorimotor gating in general, can be useful to determine the effects of genetic mutations and/or alterations in pharmacology within the circuit generating the PPI response.

6. Eph Receptors & ephrin Ligands

6.1 Molecular Biology of Eph/ephrin Proteins

Eph proteins are a receptor of the tyrosine kinase family. Their associated ligands, the ephrins, bind to the Eph proteins to form molecular bonds. Receptors, like the Ephs, are membrane bound proteins found on the surface of cells. Ephs are bound to the cell membrane by ways of a glycophosphatidylinositol (GPI) linkage. If receptors receive stimulation they cause a cascade of intracellular activity. Stimulation that could cause this intracellular response would include the binding of an associated ligand, for which the

receptor has an affinity. Receptor tyrosine kinases, specifically, have patterns of affinity and dis-affinity where Eph receptors can bind with ephrin ligands (Eph-to-ephrin, reverse interaction), and ephrin ligands can bind with Eph receptors (ephrin-to-Eph, forward interaction), (Cramer, 2005). This bidirectional signaling is specific to the Eph/ephrin proteins and adds to the diversity of Eph protein function in development (Cramer, 2005). There are A and B classes of the Eph/ephrin proteins based on the cellular membrane anchors. Generally, EphA receptors will bind to ephrin-A ligands and EphB receptors will bind to ephrin-B ligands; although there is some promiscuity between these proteins whereby EphA4 also binds to ephrin-B ligands and EphB2 also binds to the ephrin-A5 ligand (Cramer, 2005).

Eph/ephrin proteins are prominent in the developing and adult nervous system. Their principal role is to guide axons within the neural system that may influence the projection of the axonal pathway (Cramer, 2005). These proteins exhibit bidirectional behaviors that can be adhesive or de-adhesive; these behaviors are specific to the Eph/ephrin proteins and serve as positional labels to guide axonal development, and topographic map formation in the auditory system, (Wallace et al., 2013**)**.

Precise roles of the Eph/ephrins in the auditory system are not yet fully understood, yet patterns in other sensory systems have lead researchers to believe that these proteins are highly important in the guidance of axons to pattern for topographic maps. More specifically, research within the visual system has shown that Eph/ephrin proteins are highly present in the development of visual axonal pathways from the periphery to the central nervous system, (Cramer, 2005). Feldheim et al. (1998, 2004)

found that EphA4 signaling is highly evident in the developing visual system in order to guide retinotopic projections to the tectum and visual thalamus (Cramer, 2005).

6.2 Eph/ephrins in the Auditory System

Recently, researchers have begun to better understand the role of Eph/ephrins in the developing auditory system. Eph/ephrin expression patterns begin in the auditory periphery where there is strong expression of EphA4, EphB3, ephrin-A3, ephrin-B2 and ephrin-B3 in the organ of Corti and spiral ganglion cells of the mouse inner ear (Pickles, 2003). Brors et al. (2003) cultured spiral ganglion neurons and studied the expression patterns and directed growth of neuronal processes toward their targets. Here, EphA4 interacted with ephrin-B2 and ephrin-B3 to mediate the repulsion of spiral ganglion neurons. This repulsion then guides the axons of the spiral ganglion neurons to a new target, therefore aiding in the guidance of afferent auditory system (Cramer, 2005). Studies in EphB2 knockout mice have proven that this protein is important in the development of the peripheral vestibular system, where EphB2 knockout mice exhibit circling behaviors (Cramer 2005; Cowan et al., 2000; Pickles, 2003).

Unfortunately, there is currently little literature on the effects of Eph/ephrin mutations within the central auditory nervous system. Current research by Gabriele et al. (2011) has been able to illustrate the importance of Eph/ephrin signaling in the mammalian IC, specifically, prior to the onset of hearing. This experiment used NeuroVue dye to label axonal projections beginning from the mouse lateral superior olive to frequency-specific layers within the central nucleus of the inferior colliculus (CNIC). This experiment also showed EphA4 and ephrin-B2 expression gradients within the tonotopic inferior colliculus (IC), specifically with protein most concentrated within the

high-frequency, or ventromedial areas of the CNIC. Results from this experiment show the involvement of EphA4 and ephrin-B2 signaling in the development of the tonotopic organization of the mouse IC. A recent publication by Wallace et al. (2013) delved into the expression patterns from the mouse LSO to the IC based on ephrin-B2 signaling in the mouse prior to the onset of hearing. This team of researchers found that, when compared to wild-type mice, ephrin-B2^{lacZ} (mice lacking only a portion of the ephrin-B2 protein) did not display a reliable topographic pattern in lateral superior olive (LSO) to IC projections. Unlike normal Eph/ephrin signaling, mice with this particular lacZ mutation can only forward signal (ephrin to Eph), but not reverse signal (Eph to ephrin). Therefore, they concluded that ephrin-B2 reverse signaling must be fully functional for normal axonal projection mapping.

As illustrated by Gabriele (2011) and Wallace (2013), Eph/ephrin proteins are important for the frequency organization and tonotopic maps beginning in the auditory periphery and extending to the central auditory nervous system. Specifically, Eph/ephrin signaling facilitates topographic maps in the central auditory nervous system prior to the onset of cochlear development and functional hearing (Gabriele et al., 2011). Therefore, it is thought that these proteins are important for the initial set-up of the afferent projections and the input of sensory information to the system will further refine and sharpen the topographic arrangement of neurons (Mark Gabriele, Ph.D., personal communication). This evidence illustrates the continued importance of the expression of the Eph/ephrin proteins in the upper brainstem of the mouse that will have different effects on the PPI paradigm utilized in this experiment.

7. Goal of Current Experiment

To the best of our knowledge, there is currently a lack of literature utilizing the PPI paradigm to understand the effects of the Eph/ephrin proteins on the mouse auditory system, therefore the aim of this experiment was to better understand the effects of the Eph/ephrin proteins on auditory afferent development by comparing wild-type mice to mutant mice lacking an entire or partial Eph/ephrin protein. More specifically, evidence has suggested that the IC is highly involved in the PPI paradigm. Mice lacking Eph/ephrin proteins may have altered frequency structures within the IC that may disrupt the PPI response. It is our desire to show, in fact, that mice with Eph/ephrin genetic mutations have altered PPI behavior. Mice used in this experiment were of the C57BL/6J strain bred at Jackson Laboratories in Bar Harbor, ME. The control strain used were purely C57BL/6J while the EphA4^{lacZ} mutant mice were of a C57BL/6J strain base and the ephrin-B3^{null} strain was of a CD1 background. The mutant mice used in this study were EphA4 mutants, all heterozygotes lacking only one allele of the EphA4 (EphA4^{lacZ}) gene, and ephrin-B3^{null} mutants, two of which were homozygotes lacking the full ephrin-B3 protein and two of which were heterozygotes lacking only one mutant ephrin-B3 allele.

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