Samantha Luk was invited by Professor Cramer to work in her lab. A highlight of Samantha’s research experience was her development of an understanding of what it is like to be a researcher. Through her participation in all of the steps of her project, she developed independence, organization, leadership, and networking skills that will help her throughout her future career. Samantha was very active outside of her classes at UCI, serving as Co-Vice President of UNICEF at UCI, Treasurer of WHOS (World Health of Students), and Co-President of the Neurobiology Club.

Brain functions, including perception and behavior, depend critically on accurately ordered connections between nerve cells. A principle goal of developmental neuroscience is to determine how these connections form during embryonic and postnatal development. This can be studied in the auditory system, which has several precise pathways. One auditory pathway that is necessary for sound localization is found between the ventral cochlear nucleus (VCN) and the medial nucleus of the trapezoid body (MNTB). Based on previous studies of ephrin-B2, we hypothesized that this ephrin protein plays an important part in creating the specific axonal connections between the VCN and the MNTB. We investigated this hypothesis by studying the anatomy of this pathway. Fluorescent dye was used to trace the axonal connections from the VCN to the MNTB in wild type mice and mutant ephrin-B2 mice. Normally, the axons coming from the VCN project primarily to the contralateral, or opposite, MNTB. However, it was found that the mutant ephrin-B2 mice formed abnormal axonal connections from the VCN to the ipsilateral (same side) MNTB. This abnormality in the auditory pathway suggests an inhibitory role for ephrin-B2 in axonal guidance during the development of the auditory system.
Introduction

Eph receptor tyrosine kinases and their ephrin ligands are a large family of signaling proteins that have attracted particular interest due to their roles in neuronal development (Lemke 1997) and complex molecular interactions. This protein family has been found to influence important developmental processes such as axon guidance, axon targeting (Drescher et al. 1995; Cramer et al. 2006), and synapse formation (Kayser et al. 2006). As a result, Eph proteins help refine the neural systems and create precise circuitry. However, the exact functions within these processes of many of these proteins are still unknown, even though we understand their general binding patterns. Usually, ephrin-A ligands, which include ephrin-A1 to ephrin-A5, bind to EphA receptors, which include EphA1 to EphA10. Ephrin-B ligands (ephrin-B1 to ephrin-B3), bind to EphB receptors (EphB1 to EphB6) (Lemke 1997). Any ephrin-A ligand can bind with any EphA receptor, and the same goes for ephrin-B ligands with EphB receptors. However, it has been seen that EphA4 can also bind to ephrin-B2 and ephrin-B3 (Gale et al. 1997), while EphB2 can also bind to ephrin-A5 (Himanen et al. 2004). Since the ephrin ligand can bind and interact with such a wide variety of receptors, it exerts great developmental influence in the brain.

A brain area of particular interest is the auditory system, which must localize sounds in space and integrate information about the sounds’ frequency, timing and intensity. As a result of these complex demands, the auditory system needs precise connections between the various auditory structures. One such carefully regulated auditory pathway is located in the brainstem between the ventral cochlear nucleus (VCN) and the medial nucleus of the trapezoid body (MNTB) (Willard and Ryugo 1983). The MNTB is important for sound localization in mammals, including humans, because it helps process auditory information coming from both ears via the VCN (Smith et al. 1991). Interestingly, the VCN only sends axonal projections to the contralateral MNTB (Harrison and Irving 1966; Figure 1), which is on the opposite side of the brain. The axons from the VCN cross the midline, which is the middle of the brain, and terminate in calyces of Held, which are synaptic terminals that wrap around MTNB cell bodies forming a cup-like shape around the cell body (Willard and Ryugo 1983). Few, if any, projections terminate in the ipsilateral—on the same side—MNTB (Harrison and Irving 1966), which raises the question of how the axons coming from the VCN bypass the ipsilateral MNTB and preferentially terminate on the contralateral MNTB.

Figure 1
Simplified schematic of the auditory pathways in the brainstem from the ventral cochlear nucleus (VCN) to the contralateral medial nucleus of the trapezoid body (MNTB) viewed coronally (the schematic was generously lent by Karina Cramer).

We hypothesized that the axons coming from the VCN are guided by Eph receptors and ephrin ligands, specifically ephrin-B2, an ephrin protein with an unknown specific function. Ephrin-B2 is a promising ephrin candidate because it has been shown to be expressed in the cochlear nucleus, which includes the VCN, during development (Miko et al. 2007). In addition, ephrin-B2 interacts with the EphB receptors and the EphA4 receptor (Gale et al. 1996), all of which have been shown to affect axonal projections. More specifically, the EphB2 receptor has been found to regulate axon projections crossing the midline of the brain (Cramer et al. 2006), while the EphA4 receptor has been shown to regulate axon guidance in many areas (Gale et al. 1997; Brors et al. 2003), including the auditory system (Cramer et al. 2004; Hsieh et al. 2007; Huffman and Cramer 2007). Recently, it has also been discovered that mutant heterozygous mice with a mutation in one copy of the ephrin-B2 gene (ephrin-B2+/-), and a subsequent decrease in functional ephrin-B2 protein expression, had an increased hearing threshold in their auditory brainstem responses (ABRs) (Miko et al. 2008). The types of auditory responses in the ephrin-B2 mutants suggest that the major auditory impairment occurs in the connections between the cochlear nucleus and the superior olivary complex, which contains the MNTB (Willard and Ryugo 1983). Based on the known roles of ephrin-B2 and these recent findings about the auditory effects of decreased functioning ephrin-B2 levels, we suspected that ephrin-B2 may play an integral role in the development of this pathway.

To address the suspected function of ephrin-B2 in the auditory system, we compared the axonal connections from the VCN to the MNTB between normal, wild type and mutant ephrin-B2 mice. The axons were labeled with fluorescent
dye and the number of calyx terminations in the ipsilateral MNTB as compared to the contralateral MNTB was analyzed.

**Methods and Materials**

All experiments were carried out in accordance with the Institutional Animal Care and Use Committee at the University of California, Irvine, and were consistent with Federal guidelines. In this study, we used CD-1/129 mice that were either wild type or heterozygous for a mutation in the ephrin-B2 gene. When the mice were either 10 or 12 days old (P10 or P12), they were placed under heavy anesthesia with isofluorane. A tail clipping was taken for later PCR genotyping purposes to determine if the mouse was genetically wild type or mutant. The experimenter was blind to the genotype during the experiments to reduce potential bias and to ensure that all the brains received similar treatments and similar amounts of dye.

Brains were dissected and placed in 4% paraformaldehyde (PFA) at 4 ºC overnight. After one night in PFA, the brain was labeled with the fluorescent dye, NeuroVue Red (PTI Research, Inc., Exton, PA). This dye is lipophilic and passively diffuses into the neuronal membrane, which is made of lipids. To prepare the brain for labeling, the brain was removed from the PFA and the cerebellum was carefully dissected away without damaging the cochlear nuclei. A small piece of NeuroVue Red dye was then placed in the VCN of one side (Figure 2). The brain was transferred back into 4% PFA. The labeled brains were then incubated in 4% PFA at 37 ºC for 2 to 5 weeks to allow for dye transport to occur from the labeled VCN to the contralateral MNTB. After dye transport, the brains were rinsed and cut coronally into 100 μm sections with a Vibratome. These sections were mounted on glass slides and coverslipped with Glycergel to keep the sections hydrated and prevent bleaching of the fluorescence.

The brain sections were then imaged under epifluorescence using a Zeiss Axioskop microscope to see the transport of the NeuroVue dye. Images were taken digitally with an Axiocam camera and Openlab software. We focused on the axonal connections from the labeled VCN to its usual target, the contralateral MNTB, and its abnormal target, the ipsilateral MNTB. Within the MNTB we imaged individual calyces of Held to determine the number of connections made from the labeled VCN to the MNTB. Calyces were counted in every section containing MNTB in both the contralateral and the ipsilateral MNTB. Calyces were also counted throughout the thickness of each section at different levels. Brains were included in the analysis if the animal had at least five sections in which MNTB appeared. A calyx was counted if it covered at least one-third of the MNTB cell body.

A ratio of ipsilateral calyces to contralateral calyces (defined as the I/C ratio) was generated for each animal and was based on the total count for all sections in a given brain. This ratio normalized our counts for variations in the amount of dye inserted into the VCN (Russell and Moore 1997; Hsieh and Cramer 2006). Thus, although the amount of dye inserted may have been slightly different from animal to animal, the ratio created an absolute quantitative measure of the calyx number with respect to ipsilateral versus contralateral that did not depend on the amount of dye inserted or the actual total number of calyces counted. The I/C ratios were then compared between the two genotypes (wild type versus heterozygous) and two-tailed t-tests with an alpha value of 0.05 were performed to determine any statistical significance between the I/C ratios.

**Results**

We examined the axonal projections from the VCN to the MNTB of wild type and ephrin-B2 mutant P10 or P12 mice to identify any anatomical differences between the genotypes. Sample images of the MNTB as seen under fluorescent light are shown in Figures 3 and 4. The calyces (indicated by the arrows) are easily identifiable by their cup-like shape (Willard and Ryugo 1983).

Figure 3 shows the axonal projections and calyces in the MNTB as seen in a wild type mouse brain. In the wild type brains, it appeared that the VCN mainly projected to the contralateral MNTB target (Figure 3A, 3D, 3C, 3F), although there were still a small number of projections from the VCN to the ipsilateral MNTB (Figure 3E), which is normal.
Figures 3
The axonal projections from the VCN to the MNTB in normal, wild type mice (+/+), as labeled by fluorescent dye. The VCN projects primarily to the contralateral MNTB (MNTBc), as shown in A and D. In A and C, there are no ipsilateral connections. In D and E, there are only one or two ipsilateral connections but a large number of the axonal connections are to the contralateral MNTB. The dotted line in A and D indicates the midline of the brain. Examples of calyces are indicated by arrows. Higher magnification of the ipsilateral MNTB (MNTBi) can be seen in B and E. Higher magnification of the numerous calyces in the contralateral MNTB can be seen in C and F. The scale bars = 100 μm for all panels. The dorsal-ventral axis and site of dye seen in A apply for all panels.

Figures 4
The axonal projections from the VCN to the MNTB in ephrin-B2 heterozygous mutant mice (+/-) as labeled by fluorescent dye. There are projections to both the ipsilateral MNTB (MNTBi) and the contralateral MNTB (MNTBc), as shown in A and D. The dotted line in A and D indicates the midline of the brain. Examples of calyces are indicated by the arrows. Higher magnification of the calyces in the ipsilateral MNTB can be seen in B and E. Higher magnification of the calyces in the contralateral MNTB can be seen in C and F. The scale bars = 100 μm for all panels. The dorsal-ventral axis seen in A applies for all panels.

Figure 4 shows an example of the axonal projections seen in an ephrin-B2²⁻/⁻ mouse. Like the projections seen in the wild type mice, the ephrin-B2 mutant mice had axonal projections from the VCN that reached the normal contralateral MNTB target (Figure 4A, 4C, 4D, 4F). However, it appeared that the mutant mice also had VCN projections that reached the ipsilateral MNTB (Figure 4A, 4B, 4D, 4E).

The I/C ratios were grouped by genotype. The mean I/C ratio for the wild type mice was 0.047 ± 0.01 (± s.e.m.; n=15; Figure 5). The mean I/C ratio for the ephrin-B2²⁻/⁻ mice was 0.14 ± 0.04 (n=9; Figure 5). In a two-tailed t-test, the values were found to be significantly different (p < 0.01).

**Discussion**

The ephrin-B2 mutant mice have significantly different I/C ratios than the wild type mice. This finding implies that the axonal connections of the mutant ephrin-B2²⁻/⁻ mice in the VCN to MNTB auditory pathway have not developed normally as a result of the decreased ephrin-B2 protein function. The higher average I/C ratio of the mutant ephrin-B2 mice as compared to the wild type mice (Figure 5) suggests that the VCN in the mutant mice has more abnormal connections to the incorrect, ipsilateral MNTB. Moreover, it appears that the wild type and ephrin-B2 mutant mice have similarly shaped VCN axon terminals (Figures 3 and 4), which indicates that at least the axons in the VCN to MNTB pathway can still reach and terminate around a cell body like a normal axon. Overall, these results may indicate a role of ephrin-B2 in axon guidance in the VCN to MNTB pathway.

However, there is an outlying I/C ratio from one animal at about 0.4 in the mutant ephrin-B2²⁻/⁻ data (Figure 5). This high I/C ratio could potentially skew the data and confound the results. Yet when we ran two-tailed t-tests excluding the outlier, the difference between the two genotypes remained significant (p < 0.05). The p-value indicates that the significant difference between the I/C ratios of the ephrin-B2²⁻/⁻ and wild type mice is not
due to a skewing effect of a single high I/C ratio and that the implications drawn from these results are still relevant.

The idea that a decrease in ephrin-B2 causes incorrect connections in the axonal pathway between the VCN and its corresponding contralateral MNTB supports the known information about ephrin-B2 in the auditory system. These results suggest that ephrin-B2 may normally act to inhibit the formation of ipsilateral connections in this pathway because mice with a decrease in functional ephrin-B2 seem to have an increase in ipsilateral connections as compared to wild type mice. Additionally, ephrin-B2 is expressed in the cochlear nucleus from at least 3 to 18 days after birth (Miko et al. 2007). Thus, a mutation at the ephrin-B2 gene could affect the normal development of the cochlear nucleus and its efferent neurons. Finally, ephrin-B2 is known to interact with many Eph receptors, such as EphB2 (Lemke 1997) and EphA4 (Gale et al. 1997), which have been implicated in the precise localization of axons (Cramer et al. 2006; Huffman and Cramer 2007; Brors et al. 2003). As a result, it may not be as surprising to find that ephrin-B2 helps regulate the development of such an anatomically precise connection as the auditory pathway between the VCN and its contralateral MNTB target.

This finding advances our understanding of the function of ephrin-B2 in the auditory system and serves as a launching point for a more in-depth investigation of this signaling molecule. This experiment only used mice that were 10 or 12 days old. It is uncertain if ephrin-B2 has the same effects in the auditory system at a younger or older age, so further experimentation at ages other than P10–P12 would be illuminating. Perhaps ephrin-B2 is needed throughout the time of development for the correct axonal targeting in the VCN to MNTB pathway, or ephrin-B2 may only be needed during the studied time. It is also uncertain whether the abnormal connections seen in the mutant P10 and P12 ephrin-B2+/- mice are corrected at later stages of development. In addition, there are different levels of other biological molecules in the brain at different stages of development. These molecules can also affect how ephrin-B2 works in the auditory system. By identifying the time frame in which ephrin-B2 affects the auditory system, especially in this VCN to MNTB pathway, we can get a better idea of its function within this system and potential molecules with which ephrin-B2 interacts during normal development.

Furthermore, we should investigate the functionality of the axons within the VCN to MNTB pathway. Although there does not appear to be a difference in the morphology of the calyx between the wild types and the mutants or between contralateral and ipsilateral MNTB connections (Figures 3 and 4), these anatomical studies do not address the physiological integrity of these connections. We do not know if these connections are functioning as they should normally. It has been shown that Eph proteins can interact with the processes involved with myelin (Goldshmit et al. 2006), the coating that covers most neurons and increases conduction velocity. As a result, the axons projecting from the VCN might be structurally or functionally different in the ephrin-B2 mutants. In addition, the VCN axons that innervate the ipsilateral MNTB instead of the contralateral MNTB might also have structural or functional differences from the axons projecting from the VCN in wild type mice. These potential differences could affect the way that the auditory system develops and operates. Thus, further experimentation would clarify ephrin-B2’s role in this pathway.

The developing brain requires many different protein factors that interact with one another to create functionally correct pathways. The auditory pathway in the brain is no exception to this rule, as shown by the significant alteration of the axonal connections between the VCN and the MNTB as a

Figure 5
The quantification of the ipsilateral to contralateral projections from the MNTB to the VCN in ephrin-B2 mice is expressed as I/C ratios and indicates that there is a significant difference between the wild type ephrin-B2 (ephrin-B2 +/+ ) as compared to the heterozygous ephrin-B2 mutant (ephrin-B2 +/-) (p < 0.01). The error bars are shown with the horizontal line indicating the mean.
result of a mutation in the ephrin-B2 gene (Figure 5). This finding supports known information about the role of the Eph family in axon guidance (Drescher et al. 1995; Cramer et al. 2006; Hsieh et al. 2007). It also more precisely defines the role of ephrin-B2 in the development of the auditory system, suggesting that ephrin-B2 acts as an axon guidance molecule in the auditory system. Perhaps it acts specifically as an inhibitory molecule to prevent ipsilateral connections from VCN to MNTB. As a result, this finding opens the door for more experiments to further define the exact function of ephrin-B2 in the auditory system. By understanding ephrin-B2’s developmental time window and its specific method of operation within the auditory system, we can also begin to pry apart the complex protein interactions that ephrin-B2 participates in to contribute to the normal and abnormal development of the auditory system in the brain.

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**Works Cited**


