The Influence of Forced Expression of a Neurotrophic Factor on Spiral Ganglion Cells in a Presbycusis Model

by

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Abstract

Age related hearing loss is often the product of hair cell loss. The only current treatment for deafness due to total hair cell loss is direct stimulation of spiral ganglion neurons (SGNs) via a cochlear implant. However, the absence of hair cells is thought to cause death of SGNs due to a loss of neurotrophic factors being secreted from the hair cells. Previous studies have demonstrated that brain derived neurotrophic factor (BDNF) prevents SGN loss in artificially deafened animals. This study tested the hypothesis that BDNF would also prevent SGN loss associated with age related deafness in C57BL/6 mice, which begin SGN loss at 7 months of age. To investigate this, I inoculated an adeno-associated viral vector containing a BDNF gene construct into the inner ear of 9 month old mice to force prolonged expression of BDNF. As a control, I inoculated other mice with an empty virus. I assessed SGN cell density 3 months post-inoculation. Contrary to the hypothesis, the results demonstrated a decrease in SGN cell density of BDNF treated animals relative to controls. This result suggests that the concentration of BDNF reached levels that are toxic to SGNs. Further studies should be done to understand the relationship between BDNF concentration and SGN survival.

Introduction

Among the elderly population, the common disorder of progressive sensorineural hearing loss, called presbycusis, increases the difficulty of daily life for not only those affected but also for their loved ones. This age-related decline in hearing is usually a result of the degeneration of the auditory sensory receptor hair cells of the organ of Corti and/or the loss of spiral ganglion (SGN) neuron cell bodies, whose neurons synapse onto the hair cells (Willott, 1991). This results in loss of innervation of the basilar membrane and subsequent loss of auditory signal transmission to the

brain. The loss of SGN cells is thought to follow the loss of the organ of Corti (Shibata, et al., 2010).

In mammals, degeneration of auditory hair cells and neuronal cells is permanent and no spontaneous regeneration can be observed (Shibata, et al., 2010). Furthermore, as of today, the treatment for this kind of profound hearing loss is limited to surgical insertion of a cochlear implant. The cochlear implant bypasses the absent or non-functioning sensory hair cells to directly stimulate the auditory nerve (Chikar, et al., 2008). Therefore it is reasonable to conclude that the cochlear prosthesis relies on the presence of functional spiral ganglion neurons to be successful, and developing means of preventing spiral ganglion cell loss has immediate clinical interest.

Currently, a common animal model for presbycusis is the C57BL/6 mouse. This strain of mice is homozygous for the age-related hearing loss gene (ahl/ahl) a spontaneous mutation, reproduced through inbreeding, which dictates accelerated hair cell and spiral ganglion cell loss in the cochlea (Keithley et al., 2004). Loss of high frequency (greater than 20kHz) hearing sensitivity is significant by 6 months of age; reaching all frequencies by 15 months. Complete hearing loss can be found in the C57BL/6 mice by 24 months (Willott, 1991). Organ of Corti degeneration is observed between 6 and 12 months of age, being most pronounced in the basal turn (highest frequency). By 2 years, the organ of Corti region is devoid of most recognizable structures (Willott, 1991). The SGN cell density within the Rosenthal's canals (Figure 1) at 7 months of age, ranges from 80%- 100% of 1.5 month old density, from base to apex respectively. By 12 months, SGN density ranges from 40% to 85% of 1.5 month old density and 10% to 45% by 2

years (Willott, 1991). The C57BL/6 phenotype is comparable to the phenotype of humans with presbycusis, in the form of a degenerated basal turn (Keithley et al., 2004).

Neurotrophic factors, like brain derived neurotrophic factor (BDNF), are growth factor proteins that are essential to the early development and maintenance of auditory neurons in the inner ear (Shibata, et al., 2010). Previous studies by Staecker et al. (1996) and Nakaizumi et al. (2004) have shown that, if hair cells are eliminated by ototoxic drugs or by noise exposure, then spiral ganglion cells degenerate. Their death is either due to a lack of neurotrophic maintenance or a lack of activity. Additionally, they showed that forced expression of BDNF using a viral vector can preserve the SGN cells in ears with artificial hair cell loss. Other methods of BDNF inoculation, such as mini-osmotic pumps, have also demonstrated the ability preserve SGN cells in ears with artificial hair cell loss (Shibata, et al. 2010).

Viral vectors have been used as a means of forcing the transgenic expression of neurotrophic factors in the inner ear. The adeno-associated virus (AAV) has been shown to transduce outer hair cells, inner hair cells, as well as, SGN cells and other supporting cells with minimal cytotoxicity (Liu, et al., 2005). The surgical inoculation of an AAV vector featuring a BDNF gene transcript (AAV.BDNF), induces the secretion BDNF into the inner ear fluids (Shibata, et al., 2010). In this study, I used a micro-injection of AAV.BDNF, through the round window membrane (Figure 2) into the scala tympani (Figure 1) to induce transgenic over-expression of BDNF in the C57BL/6 mouse.

The goal of my study was to determine whether transgenic expression of BDNF can effectively prevent the degradation of spiral ganglion neurons in the age-related hearing loss model, C57BL/6 mice. The retention of spiral ganglion neurons is important for the function of a cochlear implant, as well as, a means for maintaining organ of Corti innervation in future regenerative studies. This study demonstrated a negative trend in spiral ganglion cell density for aged C57BL/6 mice treated with AAV.BDNF as compared to controls.

Materials and Methods

Animal care and handling and all procedures described in this work were approved by the University of Michigan Institutional Committee on the Use of Care of Animals and performed using accepted veterinary standards.

Animals and groups

This study was performed exclusively on C57BL/6 mice. To assess the effect of BDNF there was 1 treatment group and 2 control groups. Treatment animals were inoculated in the left ear with AAV.BDNF at 9 months of age and then were sacrificed 3 months post-operatively. The first control group was inoculated in the left ear with AAV.Empty at 9 months of age; then was sacrificed 3 months post-operatively. The second control group was the normal right ears of the AAV.BDNF and AAV.Empty groups.

As further controls, normal C57BL/6 mice were sacrificed at 9 months of age. A comparison of the right and left ears was performed to confirm that the right ear was a valid control for the left.

Also the 9 month old ears were compared to the 12 month old right controls to assess natural SGN cell density decline.

Viral Vectors

Adeno-associated viral vectors with a mouse *BDNF* insert driven by a cytomegalovirus promoter (AAV.*BDNF*) have been described previously (Di Polo, et al., 1998). I injected 2 µl of AAV.*BDNF* at a titer of 6×10^{15} AAV particles per ml. Control animals received an adeno-associated vector with no insert (AAV.Empty). Viral suspension was preserved at -80°C and thawed on ice before use (Shibata, et al., 2010). Previous studies indicated that AAV mediated gene expression is maintained for a minimum of 8 weeks (Duan, et al., 2002).

Surgical procedure

Vectors were injected into the scala tympani (Figure 1) using a post-auricular approach as described by Shibata. I briefly exposed the temporal bone, wherein a hole was made, exposing the base of the cochlea and the round window membrane. A plastic cannula connected to a syringe was used to puncture the round window membrane and inject 2µl of AAV.BDNF suspension into the scala tympani, the space beneath the organ of Corti.

To ensure that it is the effect of the BDNF preserving spiral ganglion cells and not an effect of the viral vector or surgical inoculation itself, control mice of the same age were inoculated using the same method with an empty viral vector (AAV.Empty). Uninjected animals at 9 and 12 mo age that received no treatment were used as normal controls to assess the amount of SGN loss over the 3 month interval.

Plastic Sections

On the animal's 3 month post-operative date, animals were deeply anesthetized and decapitated. The temporal bones were removed from the heads and partially dissected to expose the organ of Corti. Then the ears were fixed them in 4% paraformaldehyde for two hours. I then decalcified the ears in 3% EDTA, for a week, exchanging the fluid every other day. Once decalcified, I dehydrated the ears with increasing concentrations of ethanol and then embedded them in JB-4 resin. Thin sections (3 μm) were cut near the mid-modiolar plane and every 3rd section was collected to count surviving SGN.

Statistical Analysis

Photographs were taken of cross-sections of 3 Rosenthal's canals (2 basal, 1 apical) in each section for 3-6 of the collected sections, using a 20x Leica DMRB light microscope and the CCD Cooled SPOT-RT digital camera and computer program. Using the ImageJ computer program, I counted all spiral ganglion cells that contained a nucleus and calculated the canal area to assess cell density for each photographed canal. The average SGN cell density of the 9moR and the 9moL groups were compared using a 2-tailed t test to confirm that the right and left ears degenerate comparably. The average SGN cell density of the 9moR and the 12moR groups were compared using a 2 tailed t test to assess the amount of spiral ganglion loss over the course of the 3 months. The average SGN cell density of the BDNF, Empty and 12mo groups were compared using a 3-way ANOVA to assess the effect of the BDNF treatment. P value of <0.05 was considered significant. All statistics were performed using the SYSTAT computer program.

Results

Morphology

The most noticeable difference is that the SGN cells of the BDNF group appeared larger than the SGN cells of every other control group (Figure 3). Secondly, there appears to be a build up of granulated tissue, in both the scala media and the scala tympani of the BDNF treated group (Figure 3). This indicates an immune response to the secreted BDNF and not the virus because this tissue is not found in the Empty group. This also confirms that the AAV.BNDF transgenic transfer was successful. Also, hair cell loss was noted in the BDNF treated group (Figure 3) suggesting a toxic effect, which could be responsible for the immune response.

Assessing controls

The right and left side of the normal 9 month controls appear more or less the same. This was further confirmed by a 2 tailed t test with a p value of 0.803; confirming that the right ear is a valid control for the left ear.

Unfortunately, the normal 9 month old controls also appear to have the same morphology as the normal 12 month old controls. This observation is confirmed by a 2 tailed t test with a p value of 0.154. Therefore it is not valid to expect a change in the SGN cell density based on protective effects. This indicates that 3 months is not enough time to produce a significant decrease in SGN cells.

SGN cell density of BDNF group

Within any and all groups, there was no statistical difference in the upper base and the apex. The Univariate F Test comparing the BDNF, Empty and 12 month groups in just the upper base and apex gave the p values of 0.175 and 0.461 respectively.

Just looking at the mean SGN cell density in the base, it appears that there is a difference between the AVV.BDNF group and the normal 12 month control group (Figure 4). The slight decrease in the AVV.Empty group can most likely be attributed to mechanical injection damage. The Univariate F Test comparing the 3 groups in the lower base was not statistically significant (p value of 0.0794). However, a 2 tail t test comparing just the BDNF and 12 month group does produce a statistically significant difference (p value of 0.0361).

Discussion

This study assessed the outcome of forced over-expression of BNDF in the presbycusis model C57BL6 mouse by surgical inoculation of the AAV.BDNF viral vector. This forces transgenic expression and secretion of BNDF into the scala tympanic cochlear fluid. Despite previous studies that demonstrate BNDF protects the spiral ganglion cells (Shibata, et al., 2010), my BNDF treated group displayed a decreased SGN cell density in the lower basal canal (closest to the injection site) as compared to both the Empty and 12mo control groups.

Furthermore, it was demonstrated that there was no significant SGN cell loss over the course of the 3 months between the 9 month old normal controls and the 12 month old normal controls. Without SGN cell loss, the BDNF had nothing to protect. It would be beneficial to do longer term studies to assess the effect of BDNF over a time period that does have statistically significant SGN cell loss.

BDNF over-expression needs to be more finely regulated

The AVV.BDNF over-expression of this study used the titer of 6×10^{15} AAV particles /ml and demonstrated a negative trend in SGN cell density in the BDNF treated groups as compared to the control groups. Previous studies, whose results demonstrated a positive, protective effect of BDNF over-expression on the SGN cells; as seen in the Shibata study, used a titer of 4×10^{12} Ad.BDNF particles per ml in a neomycin-deafened guinea pig model and the Agterberg study which used a mini-osmotic pump filled with 100ug/ul concentration of BDNF solution. Concentration of BDNF from viral vectors is dependent on transduction efficiency, which includes initial viral binding, entry and post entry processes (Liu, et al., 2005).
Because the AAV.Empty did not produce as negative of a trend as the AAV.BDNF, the difference of my study suggests that the protective effects of BDNF are limited to a lower range of concentrations (assuming that titer is correlative to concentration). Therefore it would be worthwhile and beneficial to investigate the effect of various concentrations of AAV.BDNF on the SGN cell density in the C57BL/6 mouse.

Mechanism of hearing loss in C57BL6 mice

The C57BL/6 mice are homozygous for the age-related hearing loss gene (ahl/ahl). Complete deafening of these mice is much more gradual (over the course of 24 months) than that of artificial hearing loss (within 1 week). Therefore it would be worthwhile to investigate whether the negative trend result of spiral ganglion cell density that I observed in my study is specific to C57BL/6 mice, as well as, studies to elucidate the mechanism of action for the different deafening procedures. Further studies should include a recreation of my study in artificially

deafened guinea pigs (the model which has been more extensively studied) and artificially deafened mice to note whether the negative trend is specific to the C57BL6 mice or the titer of AAV.BDNF.

Conclusions

This study shows that, despite the literature, inoculation of AAV.BDNF in the presbycusis model C57BL/6 mice negatively affects the spiral ganglion density. This outcome may be caused by one or more of the following possibilities. A. in mice, BDNF is less efficient than other neurotrophins in protecting auditory neurons. B. The influence of neurotrophins is diminished in older ears. C. Granulation tissues and other side effect caused by BDNF may negatively influence auditory nerve survival. Further studies must be done to infer the mechanism in which BDNF affects C57BL/6 mice, as well as, which BDNF inoculation methods and concentrations are most applicable for future clinical therapies.

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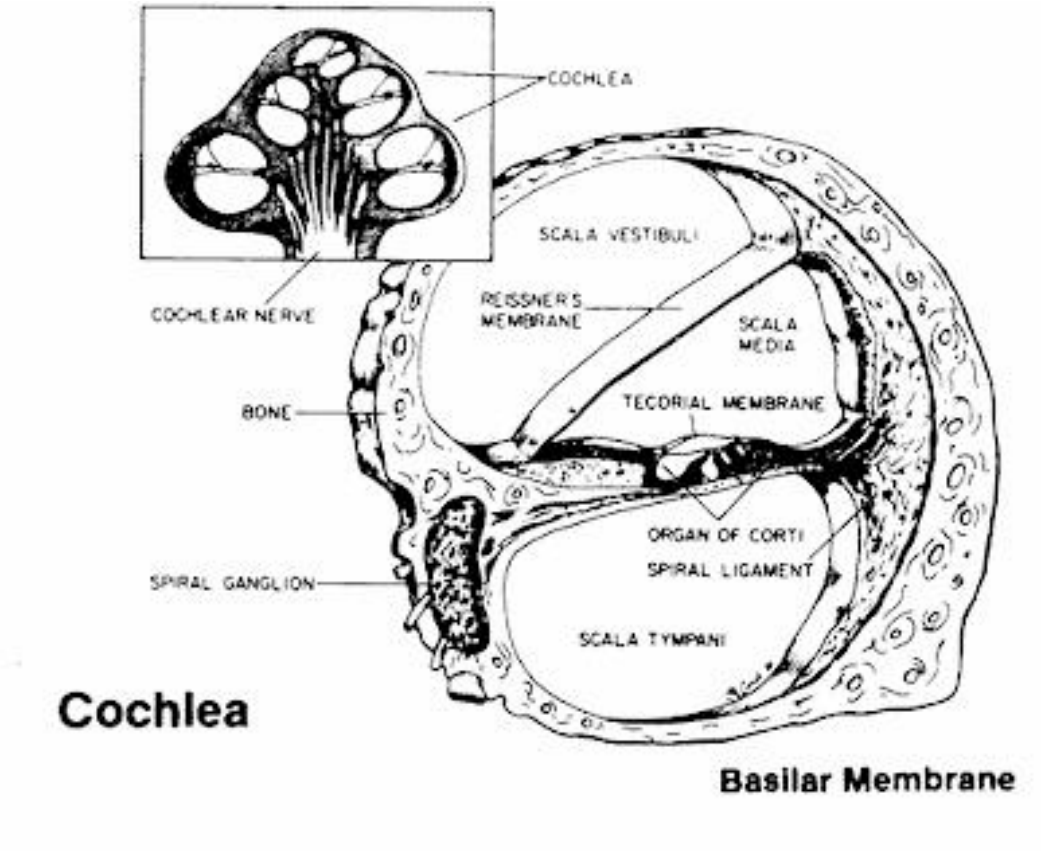

Figure 1. Illustration of the normal cochlea. The Rosenthal's canal is the oval shaded area that contains the spiral ganglion. Inset photo on top illustrates a cross section of whole cochlea with 5 sections of organ of Corti, each containing a section of Rosenthal's canal.

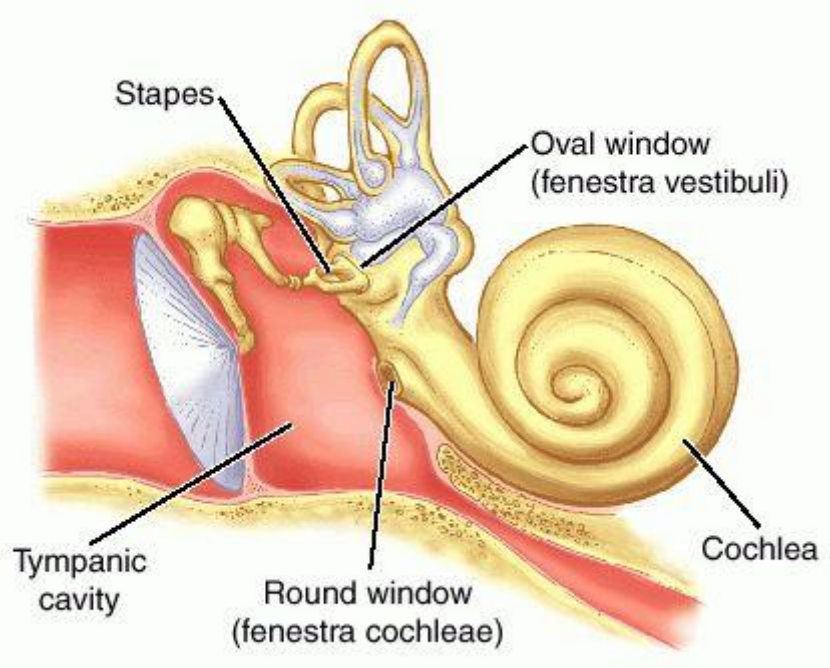

Figure 2. Illustration of the top down view of the cochlea with a view of the middle ear space. The stapes transmits mechanical sound energy into fluid waves within the cochlea via the oval window. Once energy has traveled the up the apex via the scala vestibule and back down the connecting scala tympani, energy is dampened by the round window membrane (Willott, 1991).

Table 1.

Summary of groups

Group I (n=4)	Treatment	AAV.BDNF inoculated left ear at 9 months of age. Sacrificed at 12 months of age.
Group II (n=4)	Control	AAV.Empty inoculated left ear at 9 months of age. Sacrificed at 12 months of age.
Group III (n=4)	Control	Normal right ear. Sacrificed at 12 months of age.
Group IV (n=4)	Control	Normal left ear. Sacrificed at 9 months of age.
Group V (n=4)	Control	Normal right ear. Sacrificed at 9 months of age.

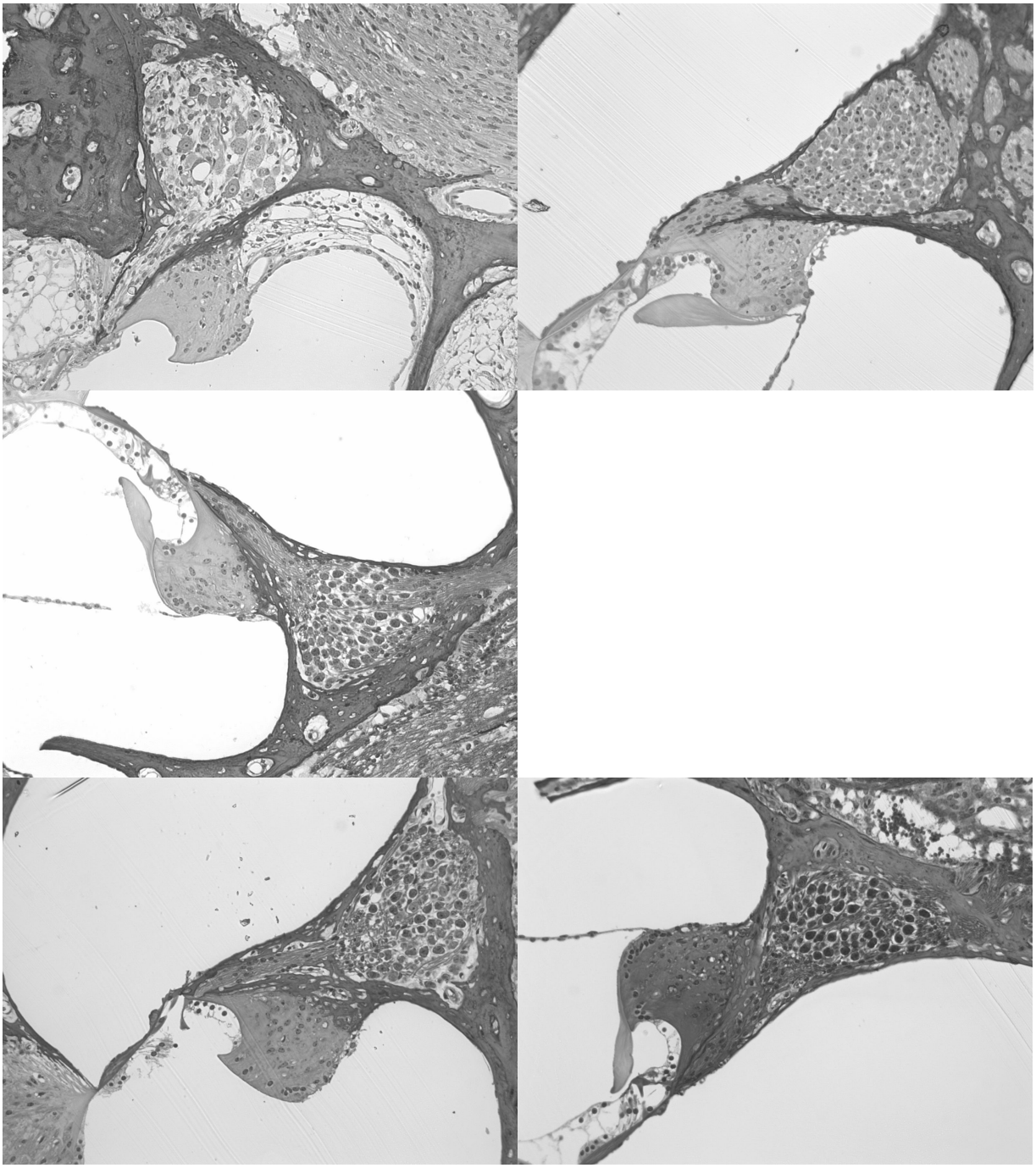

Figure 3. Top left: AVV.BDNF. Top right: AVV.Empty. Middle: normal 12 month. Bottom left: normal 9 month left. Bottom right: normal 9 month right. All photos are of the lower base.

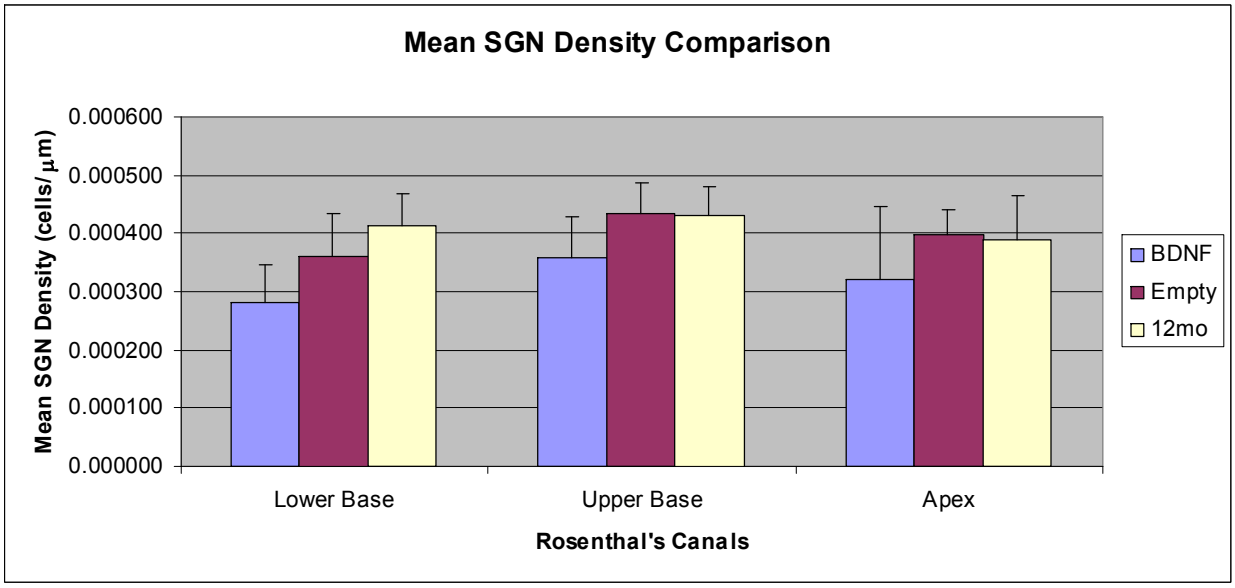

Figure 4. Bar graph of the mean of the spiral ganglion densities for each group. Error bars represent standard deviation of the mean.