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# The Effect of Noise Exposure on Inhibitory Neurotransmission in the Auditory, Visual and Multisensory Cortices in Rats

Sarah Fitzpatrick

Western University, [sfitzpa9@uwo.ca](mailto:sfitzpa9@uwo.ca)

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THE EFFECT OF NOISE EXPOSURE ON INHIBITORY NEUROTRANSMISSION IN THE AUDITORY,  
VISUAL AND MULTISENSORY CORTICES IN RATS

(Thesis format: Monograph)

by

Sarah Fitzpatrick

Graduate Program in Anatomy and Cell Biology

A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Master of Science

The School of Graduate and Postdoctoral Studies  
The University of Western Ontario  
London, Ontario, Canada

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## Abstract

It is well established that high-intensity noise exposure can induce structural and physiological changes in the primary auditory cortex, such as impaired GABA neurotransmission, which leads to a reduced level of GABA-synthesizing enzymes (i.e., GAD65/67). At present, however, it remains unknown how partial hearing loss affects GABA neurotransmission in areas of the cortex that process sound as well as other sensory modalities (e.g., visual stimuli). In the present study, we are using a rat model to investigate our working hypothesis that noise-induced hearing loss causes a differential effect on GAD67 levels in the various cortical areas capable of sound processing, such that the multisensory cortex will not show the same dramatic reduction as in the primary auditory cortex. Adult male rats were exposed to loud noise (0.8-20 kHz at 120 dB SPL for 2h), which caused a  $27 \pm 9$  dB elevation of their hearing threshold 14 days following the noise exposure. Using immunohistochemistry, the level of GAD67 was compared between noise-exposed rats and age-matched controls in multiple auditory, visual and multisensory cortical areas. Consistent with our hypothesis, preliminary findings suggest that the multisensory cortex does not experience the same degree of impaired GABA neurotransmission as the primary auditory cortex following noise-induced hearing loss.

## Keywords

Noise exposure, partial hearing loss, GABA neurotransmission, GAD67, primary auditory cortex, multisensory cortex, rat model, immunohistochemistry

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# 1 Literature Review

## 1.1 Noise Exposure

Overexposure to loud noise can be very damaging to the auditory system. Both the level of noise and the duration of exposure are influential factors to the degree of damage. Noise levels are measured in decibels (dB), the higher the decibel level, the louder the noise. So, how loud is too loud? A sound over 85 dB, equivalent to the sound of a blow dryer or kitchen blender, is enough to result in permanent noise-induced hearing loss (Hearing and Balance, 2014).

Subsequently, for every 3 dB's over 85 dB, the exposure time before damage can occur is cut in half (Hearing and Balance, 2014). Now, the questions become: how do we hear sound and by what mechanism does loud noise exposure damage the auditory system?

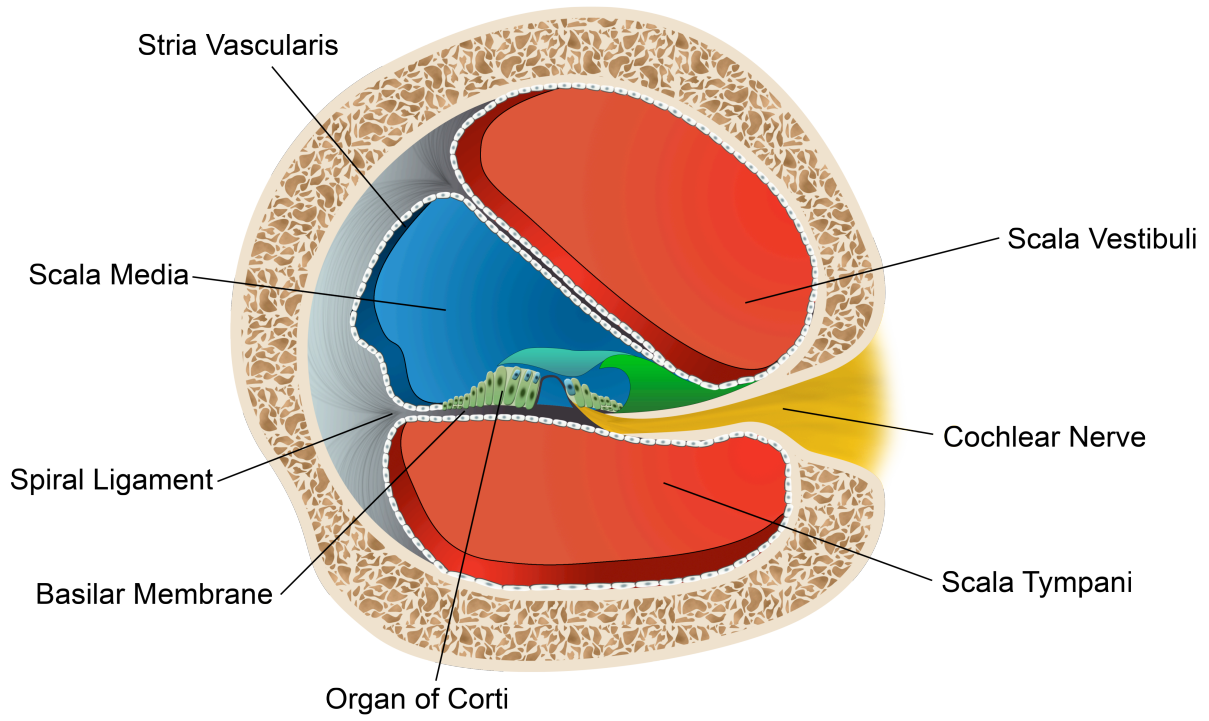
## 1.2 Sound Processing in the Peripheral Auditory System

The human ear is divided into three cavities – the outer, middle, and inner ear. The latter contains the cochlea, which is the auditory portion of the ear. The cochlea is a spiral-shaped, hollow bone that houses three fluid chambers. The first chamber, scala vestibuli, contains a watery liquid, perilymph, and comes in contact with the oval window, a membrane that connects the inner ear to the middle ear. The second chamber, scala tympani, also contains perilymph and ends at the round window, which is a flexible membrane that allows fluid in the cochlea to move. The final chamber, scala media, is the region containing endolymph and it lies in between the other two chambers (Inner Ear Anatomy, 2013). The organ of Corti, the sensory organ of

hearing, is located along the partition separating scala media from scala tympani. This partition is called the basilar membrane. The organ of Corti is composed of a cellular layer of epithelium, in which the sensory hair cells are found. These hair cells sit on the basilar membrane and are topped with small, hair-like structures called stereocilia that project into the endolymph of scala media. Another type of specialized cell found in the organ of Corti are pillar cells. Pillar cells function as supporting cells for hair cells and they provide mechanical coupling between the basement membrane and the hair cells (Auditory System: Structure and Function, 1997). Together, these structures form the inner ear and play a critical role in the complex process of hearing [Figure 1.1].

In order to hear a sound, it must first travel through the outer and middle ear cavities to reach the oval window. When the mechanical force generated from a sound strikes the oval window, the vibrations of the membrane cause the perilymph to move. As the fluid moves, both the basilar membrane and organ of Corti move as well. In turn, thousands of hair cells sense the motion through their stereocilia. The stereocilia are then able to convert the motion into electrical signals due to the modulation of the permeability of protein channels embedded in the cell membrane surrounding the stereocilia (Patuzzi, 2002). The electrical signals then communicate with thousands of primary auditory neurons of the spiral ganglion. The primary auditory neurons then transform the signal into action potentials, which travel via the auditory nerve to higher order structures of the central nervous system, including the brainstem, thalamus and cortex, for further processing.

Auditory information leaving the cochlea and traveling to higher order structures is arranged in a very specific fashion, called tonotopy. This tonotopic map of the cochlea is ordered such that different frequencies are transmitted separately along specific parts of the basilar membrane (Auditory System: Structure and Function, 1997). The basilar membrane varies in its degree of stiffness, which is due to the thickness and width of the membrane (Camhi, 1984). The basilar membrane is stiffest near the oval window, only allowing high-frequency vibrations to move the membrane and thus the hair cells (Guenter, 1978). Further along the cochlea towards the apex, the basilar membrane becomes less stiff, thus permitting lower frequencies to move the membrane (Guenter, 1978). In this way, hair cells in the organ of Corti respond to certain sound frequencies based on their physical location within the cochlea. Additionally, each cell has a characteristic frequency, the frequency at which the cell is maximally responsive, however, cells will respond with less strength to surrounding frequencies (Eggermont and Roberts, 2004). The tonotopic organization remains linear in relation to its position in the organ of Corti as it projects to higher order structures.



**Figure 1.1** Schematic of a cross-section through the cochlea depicting the fluid chambers, basilar membrane and organ of Corti.

## 1.3 The Effect of Noise Exposure on the Peripheral Auditory System

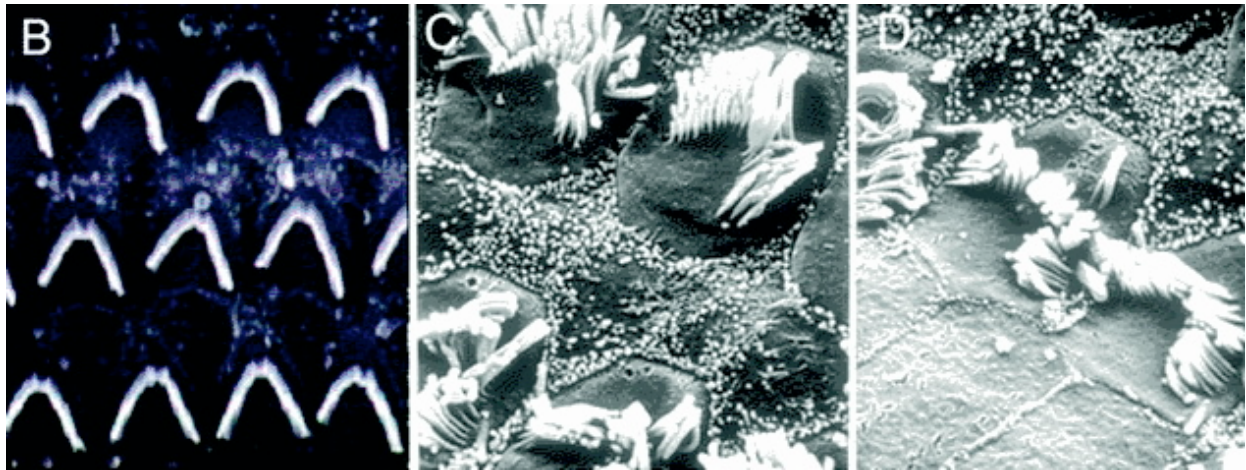
It is well established that exposure to high-intensity noise causes physical changes within the cochlea that lead to temporary or permanent hearing loss (Henderson et al., 2006). More specifically, stereocilia can be broken, fused, or a complete loss of stereocilia rootlets can occur, resulting in decreased structural integrity [Figure 1.2] (Henderson et al., 2006; Liberman, 1987). In addition, the ability of the stereocilia to convert a mechanical force into an electrical signal is reduced as a result of decreased permeability of the cell membrane surrounding the stereocilia (Patuzzi, 2002).

Noise exposure has also been shown to damage the vasculature in the peripheral auditory system. The cochlea has a rich blood supply at the spiral ganglion and along the lateral wall, referred to as the stria vascularis. Following noise exposure, acute swelling has been observed in the stria vascularis (Wang et al, 2002), which leads to a permanent loss of intermediate cells of the stria (Hirose and Liberman, 2003). Over time, the swelling disappears, but given that cell loss is permanent, the overall size of the stria vascularis decreases (Hirose and Liberman, 2003). Associated with damage to the lateral wall blood vessels, high-intensity noise exposure can also impact cochlear blood flow (Henderson et al., 2006). A reduction in cochlear blood supply, can lead to cochlear tissue damage and consequently result in hearing loss (Henderson et al., 2006).

Most cell populations in the cochlea are vulnerable to damage following noise exposure, however, hair cells are the most prominent target, and loss of these sensory cells leads to

increased hearing thresholds (Henderson et al, 2006). Depending on the length and intensity of noise exposure, the pathology can spread to include more sensory cell death, damage to the stria vascularis and eventually loss of auditory nerve fibers (Bohne, et al., 1976).

Lastly, exposure to loud noise can interfere with the tonotopic organization of the cochlea. If the pillar cells are damaged as a result of impulse noise (Salvi et al., 1979), it disturbs the local impedance of vibration, and consequently impairs the specific spatial arrangement coded in the organ of Corti (Henderson et al., 2006).



**Figure 1.2.** All images are from chinchilla cochlea samples. (B) Scanning electron microscopic image of normal outer hair cell stereocilia. (C) & (D) Scanning electron microscopic pictures of outer hair cell stereocilia following noise exposure. Image adapted from Henderson et al., 2006 with permission.

## 1.4 Manifestation of Cochlear Damage Following Noise Exposure

It is clear that overexposure to loud noise leads to physical changes in the cochlea, but the mechanism at the cellular level has yet to be addressed. It is widely accepted that oxidative stress plays a major role in this mechanism. As a result of noise exposure, there is a higher demand on the mitochondria, thus, superoxide species, an unwanted byproduct of aerobic metabolism, are also released in greater amounts (Henderson et al., 2006). The overproduced superoxide species can then react with other molecules, leading to the formation of reactive oxygen species (ROS) in the cochlea (Halliwell and Gutteridge, 1999). Ischemia in the cochlea and excitotoxicity at the junctions between hair cells and afferent auditory fibers can also result in the production of ROS (Yamane et al., 1995; Henderson et al., 2006).

ROS are capable of damaging DNA and the cell membrane, as well as breaking down amino acids, lipids and enzymes (Bayr, 2005). The end result of superfluous ROS is cell death from a combination of both necrosis and apoptosis. Necrotic cell death is a passive process, where the cell swells, ruptures and spills its contents, initiating an inflammatory response (Elmore, 2007). On the other hand, apoptotic cell death plays a normal role in aging to eliminate unwanted cells (Elmore, 2007). However, if apoptosis is initiated at the wrong time crucial cells may die. In 2002, Hu et al. were the first to report the coexistence of necrotic and apoptotic hair cells following noise exposure. Therefore, increased ROS as a result of high-intensity noise exposure leads to both necrosis and apoptosis, ultimately causing sensory cell death within the cochlea and reduced sensitivity to sound (i.e an elevated hearing threshold).



## 1.5 Noise Exposure and Hearing Loss

Noise exposure can cause temporary or permanent hearing loss. In the case of permanent hearing loss, damage occurs in the peripheral auditory system and sensitivity thresholds remain elevated. However, in the event of temporary hearing loss, threshold sensitivity recovers, which has been assumed to indicate a reversal of damage to the inner ear and no consequences for auditory function. Interestingly, Kujawa and Liberman (2009) determined that overexposure to loud noise resulting in reversible threshold elevation leaves the cochlear sensory cells intact, but causes acute loss of afferent nerve terminals and delayed degeneration of the cochlear nerve. They suggest that temporary hearing loss has progressive consequences not always identified by threshold testing, and can lead to difficulty hearing in noisy environments and possibly contribute to perceptual anomalies commonly associated with inner ear damage, such as tinnitus.

Hearing sensitivity can be assessed in two ways: behavioral threshold testing and the auditory brainstem response (ABR). For behavioral threshold testing, a sound is played and if the animal hears the sound, it is trained to respond in a particular way. If the animal does not hear the sound, it responds in a different way. This allows one to establish the specific frequencies at which an animal can hear, and ultimately determine their hearing threshold. On the other hand, an ABR uses ongoing electrical activity in the brain to extract an auditory evoked potential. A series of vertex positive waves (I through IV in rats) are recorded in the first 10 milliseconds after onset of an auditory stimulus. The auditory structures that generate waves I to IV include cranial nerve VIII, the cochlear nucleus, the superior olivary complex, and the lateral lemniscus,

respectively (Shaw, 1988). Wave amplitude (number of neurons synchronously firing) and latency (speed of transmission) are used to determine hearing threshold. In particular, Wave I amplitude closely corresponds to inner ear damage. More specifically, if Wave I amplitude decreases, even without a threshold elevation, it can indicate cochlear damage (Kujawa and Liberman, 2009). Thus, carefully assessing hearing sensitivity before and after exposure to loud noise can provide a functional assessment of the damage inflicted.

## 1.6 The Effect of Noise Exposure on the Central Auditory System

Noise-induced damage to the peripheral auditory system leads to a loss of afferent activity traveling to higher order structures of the central auditory system, including the brainstem, midbrain and cortex. In conjunction with the primary auditory cortex, the multisensory and visual cortices also process auditory information. Thus, the loss of afferent information from the peripheral auditory system disrupts normal sound processing in all of these higher order structures.

Similar to the tonotopic arrangement seen in the cochlea, there is an orderly representation of spectral frequency throughout the central auditory system, which reflects the coding of sound frequency by the basilar membrane (Eggermont and Roberts, 2004). Given this arrangement, it follows that reorganization of the cochlea, induced by noise trauma, can also lead to alteration of the tonotopic maps in the central auditory system as the afferent information travels to the cortex (Robertson and Irvine, 1989; Eggermont and Komiya, 2000).

Interestingly, specific noise exposures will result in different effects on the central auditory system. For example, a high-frequency noise exposure will alter the tonotopic organization in the auditory cortex such that cortical neurons with a characteristic frequency (CF) in the high frequency region, where hearing loss occurred, no longer respond according to their place in the tonotopic map. Instead, these neurons reflect the frequency of their less affected neighbors [Figure 1.3] (Eggermont and Roberts, 2004). This occurs as a result of an imbalance between excitation and inhibition, which can lead to an unmasking of excitatory inputs (Eggermont, 2006). Under normal conditions, the CF of a cortical neuron has the ability to inhibit the frequency regions above and below it, producing a narrow frequency-tuning curve. However, after noise-induced trauma, cortical neurons may be deprived of input near their CF and consequently, the inhibitory effects that these inputs exert become absent or greatly reduced. Thus, previously inhibited inputs can now activate the neuron at frequencies other than the original CF. Over time, this mistuning of neurons, due to the reduced activity of inhibitory neurotransmission, can change the tonotopic map of the auditory cortex (Eggermont, 2006).

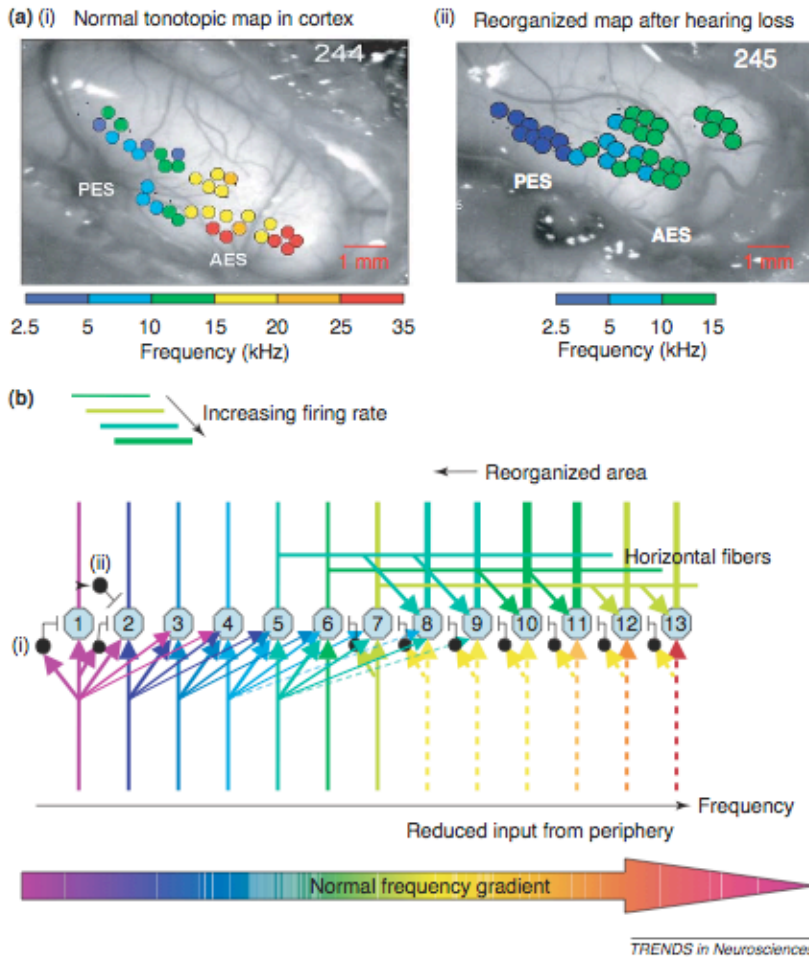
There are two types of neurotransmission in the central nervous system, inhibitory and excitatory, and a balance of these two is essential to maintain normal neuronal activity. GABA, the major inhibitory neurotransmitter, is synthesized by the decarboxylation of glutamate. Glutamic acid decarboxylase (GAD) is the rate-limiting enzyme in the catalysis of this reaction, and thus serves as a useful marker for GABA expression (Abbott et al., 1999). In addition to the role of GABA as an inhibitory neurotransmitter, GABA synthesis may have a metabolic function in energy production (Abbott et al., 1999). These two distinct roles for GABA are thought to be the reason that two GAD isoforms exist (Martin and Rinvall, 1993).

GAD isoforms of molecular weight 65,000 and 67,000 (GAD65 and GAD67) synthesize GABA at different locations in the cell and for functionally distinct purposes (Erlander and Tobin, 1991; Esclapez et al., 1994; Feldblum et al., 1993). GAD67 is spread evenly throughout the neuron, while GAD65 is localized to nerve terminals (Kaufman et al., 1991). GAD67 synthesizes GABA for neuronal activity unrelated to neurotransmission, such as synaptogenesis and protection from neural injury (Abbott et al., 1999). This function requires a widespread, ubiquitous presence of GABA. In addition, GAD67 must be active at all times for normal cellular functioning, thus it is nearly saturated with its cofactor, pyridoxal 5' -phosphate (Abbott et al., 1999). On the other hand, GAD65 synthesizes GABA for neurotransmission, thus it is only required at nerve terminals and synapses (Abbott et al., 1999). This function does not require GAD65 to be active at all times, and for this reason the majority of GAD65 is present as an apoenzyme, which is believed to be an inactive reservoir (Martin et al., 1991).

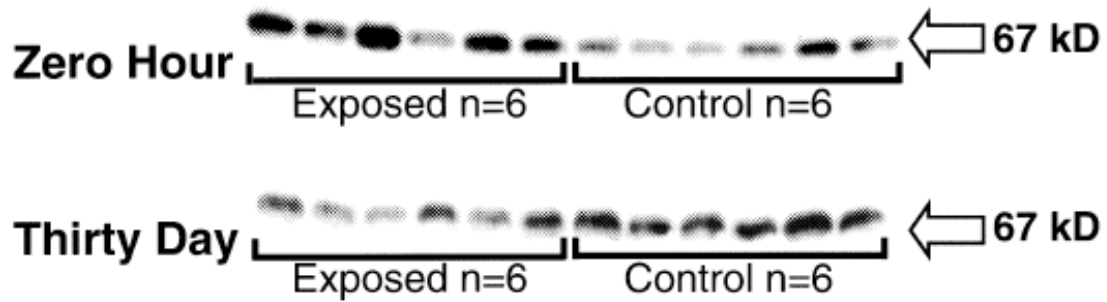
Results from previous studies indicate that noise exposure decreases GABA neurotransmission levels in the inferior colliculus (Abbott et al., 1999, Milbrandt et al., 2000, Pouyatos et al., 2003). Abbott et al. (1999) exposed three groups of rats to a 10 kHz tone at 100 dB for 9 hours. All groups were sacrificed at 0h, 2h and 30 days post exposure and GAD levels in the inferior colliculus were compared across all groups and unexposed controls. After 0 hours, there was an increased level of GAD, thought to be a defense response. After 2 hours, and 30 days, GAD levels were significantly below controls [Figure 1.4] (Abbott et al, 1999). Furthermore, Xu et al. (2009) demonstrated that when rats are noise exposed throughout development, GAD65 levels decrease in the primary auditory cortex. Moreover, Yang et al. (2011) also demonstrated a decrease in GAD65 in the primary auditory cortex, however, this was in response to a hearing

lesion. In addition, Ling et al. (2005) described a relationship between age-induced hearing loss and inhibitory neurotransmission. They showed a decrease in both GAD65 and GAD67 protein levels in the primary auditory cortex of aged rats. They postulate that this decrease in inhibitory neurotransmission is likely related to deafferentation. Given the above information, it is widely accepted that hearing loss leads to plasticity in high order auditory structures.

In view of the fact that auditory information is also processed in the multisensory and visual cortices, it is reasonable to believe that the effects of noise exposure will extend beyond the central auditory system and into these associated regions. To our knowledge, no other experiments have attempted to explain how noise-induced hearing loss affects GABA neurotransmission in areas of the cortex that process sound as well as other sensory modalities (e.g., visual stimuli). In the present study, we are using a rat model to investigate our hypothesis that noise-induced hearing loss causes a differential effect on GAD67 levels in the various cortical areas capable of sound processing, such that the multisensory cortex will not show the same dramatic reduction as in the primary auditory cortex.



**Figure 1.3.** A schematic of normal and reorganized tonotopic maps in the primary auditory cortex of cats. (a) The colours represent the characteristic frequency at each recording site, and are overlaid on the cortical surface of a control cat (i) and a cat with noise-induced hearing loss (ii). (b) The effect of high-frequency hearing loss on input to pyramidal cells (1-13) in the auditory cortex. The colour coding of the thin vertical lines reflects their frequency-specific input from the thalamus. The dashed lines are in the higher frequencies, where hearing loss occurred, and they represent the reduced ability to activate the pyramidal cells at low stimulus. Many divergent connections from each thalamic cell to a range of cortical cells are shown (lines with the same colour). Unaffected cells can impose their own frequency-selective inputs on cortical cells in the hearing loss range. Image adapted from Eggermont and Roberts (2004) with permission.



**Figure 1.4.** Western blots from analysis of GAD67 protein at 0 h and 30 days post-exposure, and their matched controls. Each lane represents two inferior colliculi from the same animal. Six noise-exposed and six controls are shown above for comparison. Image from Abbott et al. (1999) with permission.

## 2 Hypothesis and Objective

It was hypothesized that noise-induced hearing loss would cause a differential effect on GAD67 levels in the various cortical areas capable of sound processing, such that the multisensory cortex would not show the same dramatic reduction as in the primary auditory cortex.

### **Objective:**

Using immunohistochemistry, the level of GAD67 was compared between noise-exposed rats and age-matched controls in multiple auditory, visual and multisensory cortical areas.



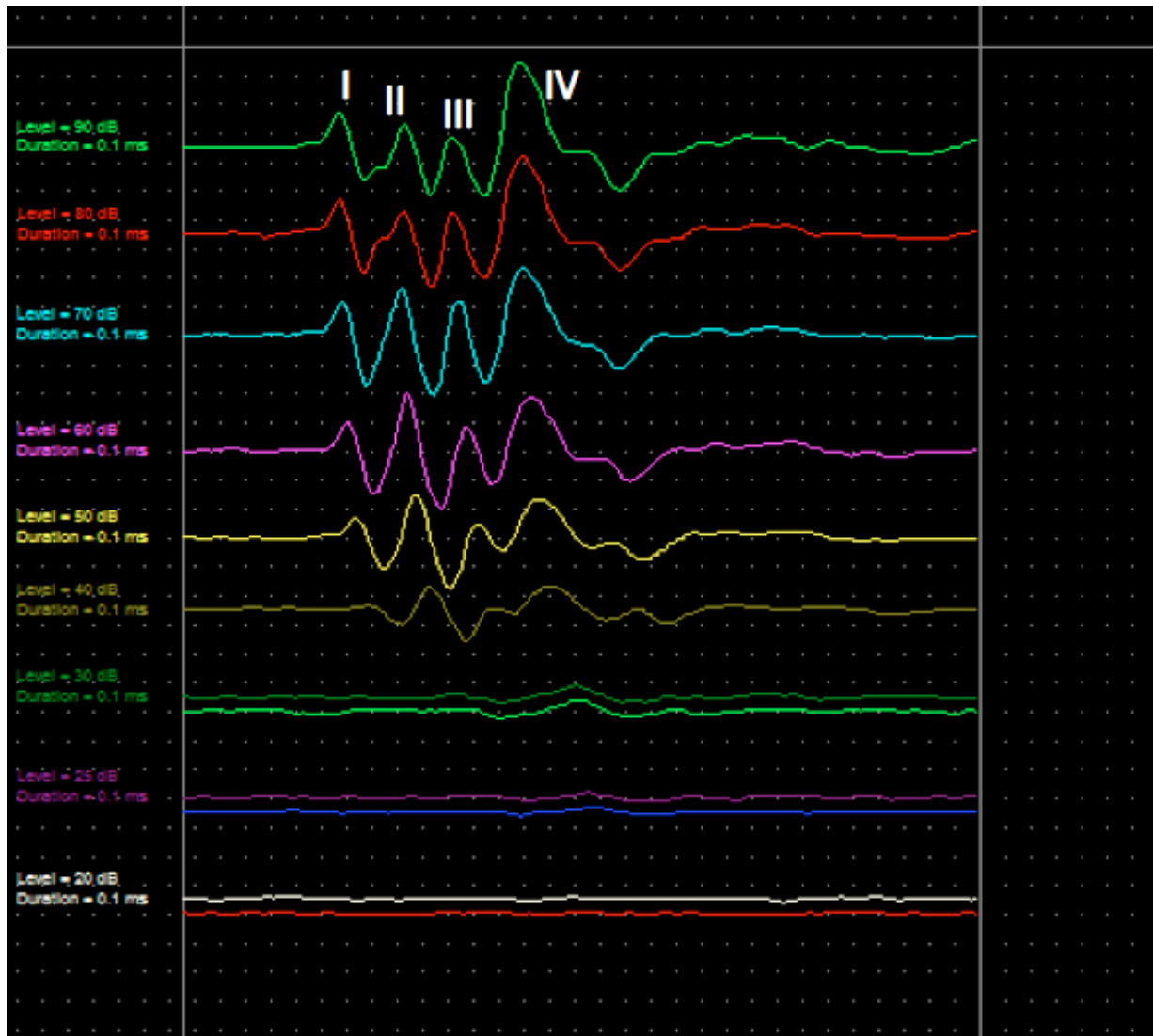
## 3 Materials and Methods

### 3.1 Animal care and handling

Ninety-day-old adult male Sprague Dawley rats, obtained from Charles River Laboratories (Senneville, Quebec, Canada), were used for this study. Animals were separated into three groups: (1) noise exposed, (2) sham and (3) control. Animals were housed at Western University in the animal care facility and kept on a 12/12-hour light/dark cycle. All experiments were conducted under protocols approved by Animal Care and Veterinary Services at Western University. Efforts were made to limit animal numbers and minimize animal suffering. For all stimulation and recording experiments, animals were deeply anesthetized with a mixture of ketamine (80mg/kg body weight) and xylazine (5mg/kg body weight).

### 3.2 Auditory Brainstem Response

An auditory brainstem response (ABR) uses ongoing electrical activity in the brain to extract an auditory evoked potential [Figure 3.1]. An ABR was performed on Day 0 and Day 14 for both control and experimental animals in order to provide a functional assessment of the damage caused by noise exposure. Thresholds for all animals were measured in response to broadband clicks and pure tones at 4 and 20 kHz. A Tucker-Davis Technologies system with BioSig software was used to record threshold measurements. Sub dermal electrodes were inserted into the back (ground), base of pinna towards the mastoid process (reference) and at the vertex (active). The high-pass filter was set at 300 Hz and the low-pass filter at 3 kHz.



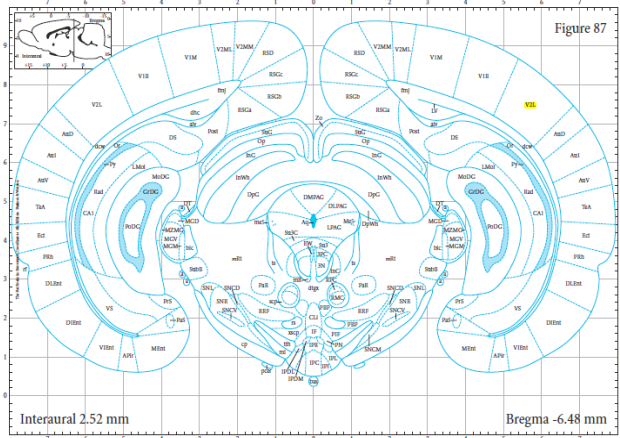
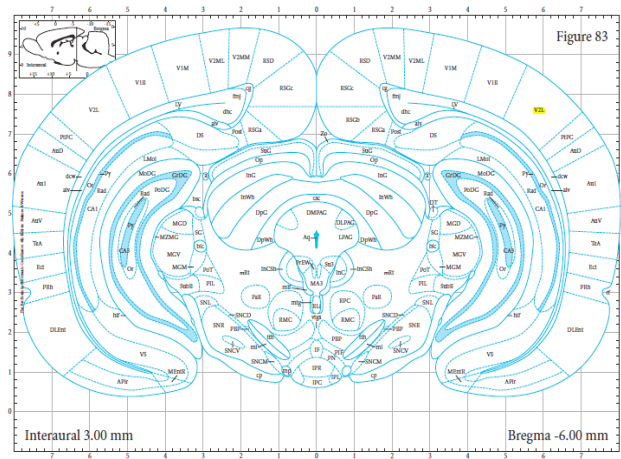
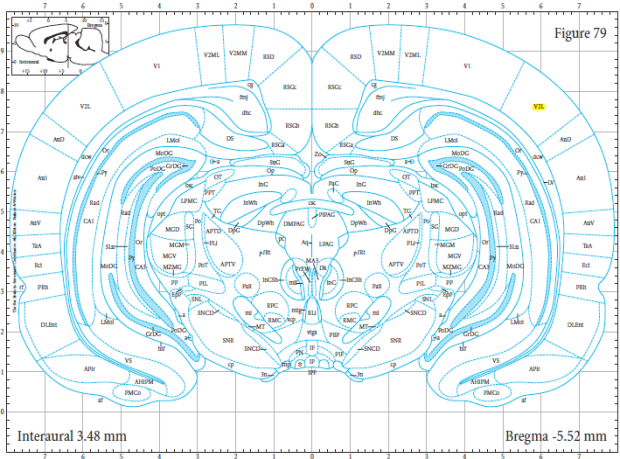
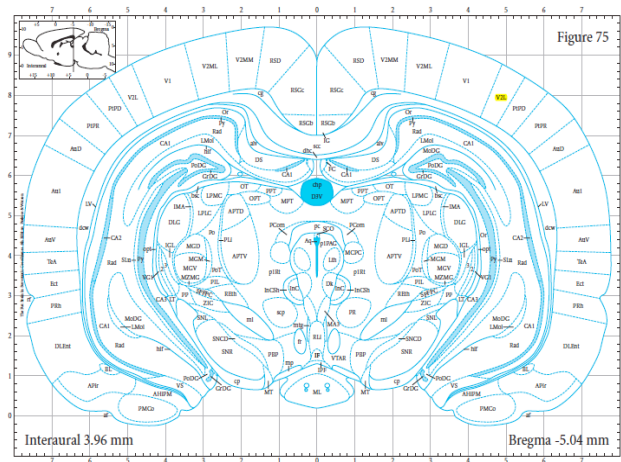
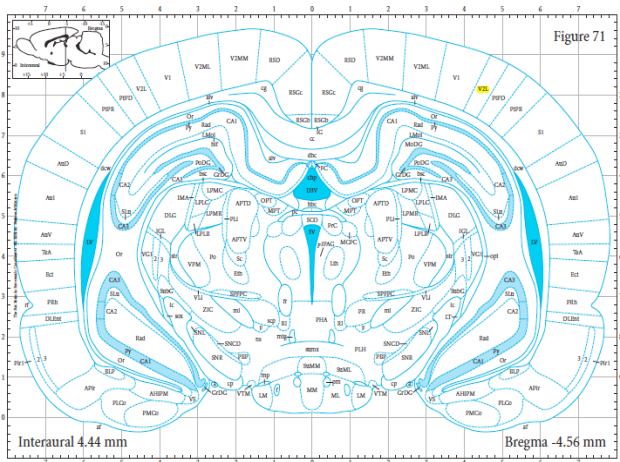
**Figure 3.1.** An ABR in response to click stimuli in a control animal. Recording of electrical activity in the brain results in a series of vertex positive waves, where wave one (I) is generated by cranial nerve VIII, wave two (II) is generated by the cochlear nucleus, wave three (III) is generated by the superior olivary complex, and wave four (IV) is generated by the lateral lemniscus, which occur within the first 10 milliseconds of stimulus onset (Shaw, 1988). Hearing threshold is reached when the waves disappear. Here, the threshold is 25 dB.

### 3.3 Noise Exposure

Animals were placed in a sound-attenuating booth with a speaker positioned 10 cm from the vertex for bilateral exposure. Animals in the experimental group were exposed to a 120 dB sound with a broadband tone between 800 Hz – 20,000 Hz for two hours. A Tucker-Davis Technologies digital-to-analog converter generated the signal. Control animals were not exposed to the auditory stimulus. This high-intensity noise exposure represents a partial hearing loss.

### 3.4 Tissue Processing

All animals were sacrificed by transcardial perfusion using 300 ml of 0.9% saline containing Heparin (1000U/ml), following by 400ml of 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB; pH 7.2). Animals were perfused 14 days following the initial ABR. Brains were harvested and post-fixed for 24 hours in the PFA mixture, after which they were immersed in 15% sucrose in 0.1M PB for 24 hours, and then transferred into 30% sucrose in 0.1M PB and stored at 4°C. Brains were sliced using a freezing microtome (KS34S, Micron, Walldorf, Germany) from bregma -4.50 mm to bregma -6.50 mm, creating 40 µm frozen coronal sections. Sections from each brain were collected in series and placed in storage solution (30% glycerol, 30% ethylene glycol, and 40% 0.1M PB) and kept at -20°C. For further analysis, only sections at bregma -4.50 mm, -5.00 mm, -5.50 mm, -6.00 mm, and -6.50 mm were used, as per Paxinos and Watson 6<sup>th</sup> Edition [Figure 3.2]. Prior to immunohistochemical staining, free-floating sections were washed (3 × 5 minutes in 0.1M PB).



**Figure 3.2.** Coronal sections from 4.5-6.5 mm caudal to bregma, as per Paxinos and Watson 6<sup>th</sup> Edition, showing our sections of interest.

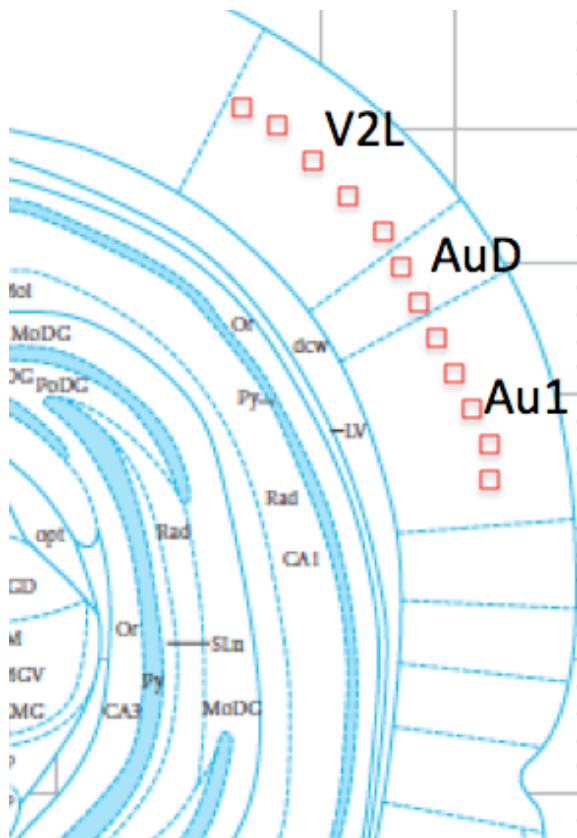
### 3.5 Immunohistochemistry

The expression of GAD67 was tested in all animals of both noise exposed, sham and control groups. All immunohistochemical incubations were performed at room temperature. Prior to GAD67 labeling, a nissl stain was performed on a series of sections from each brain. This basic dye binds to negatively charged nucleic acids and provides landmarks to determine specific brain areas, which we used as a tool to verify our region of interest. Once we had selected our sections, they were blocked with 10% goat serum (Sigma-Aldrich, St. Louis, MO, USA) and 10% bovine serum albumin, with a remainder of buffer (1ml/epindorf tube) for 1 hour to avoid non-specific binding. Subsequently, free-floating sections were washed ( $3 \times 5$  minutes in 0.1M PB), and incubated with mouse anti-GAD67 primary antibody (1:1000; MAB5406 Millipore, Billerica, MA, USA) overnight. Following primary antibody incubation, sections were washed ( $3 \times 5$  minutes in 0.1M PB) and bathed in biotinylated goat-anti mouse secondary antibody (1:500; Vector Laboratories, Burlingame, CA, USA) for 1 hour. To achieve a high level of signal amplification, sections were then washed ( $3 \times 5$  minutes in 0.1M PB) and incubated in 0.1M PB containing avidin-biotin horseradish peroxidase (1:500; Vector Laboratories, Burlingame, CA, USA) for 1 hour, followed by 0.1M PB with Biotinylated Tyramine (1:250; Perkin Elmer, Woodbridge, ON, Canada) and 3% H<sub>2</sub>O<sub>2</sub> for 10 minutes. Next, sections were incubated for 90 minutes in 0.1M PB containing Alexa Fluor (AF) 568-conjugated streptavidin (1:250; Molecular Probes, Eugene, OR, USA), which is a fluorescent dye that tags biotin. Lastly, sections were washed ( $3 \times 5$  minutes in 0.1M PB) again and mounted on glass slides and kept in the dark for 24 hours to dry. Using Vectashield mounting media (Vector Laboratories,

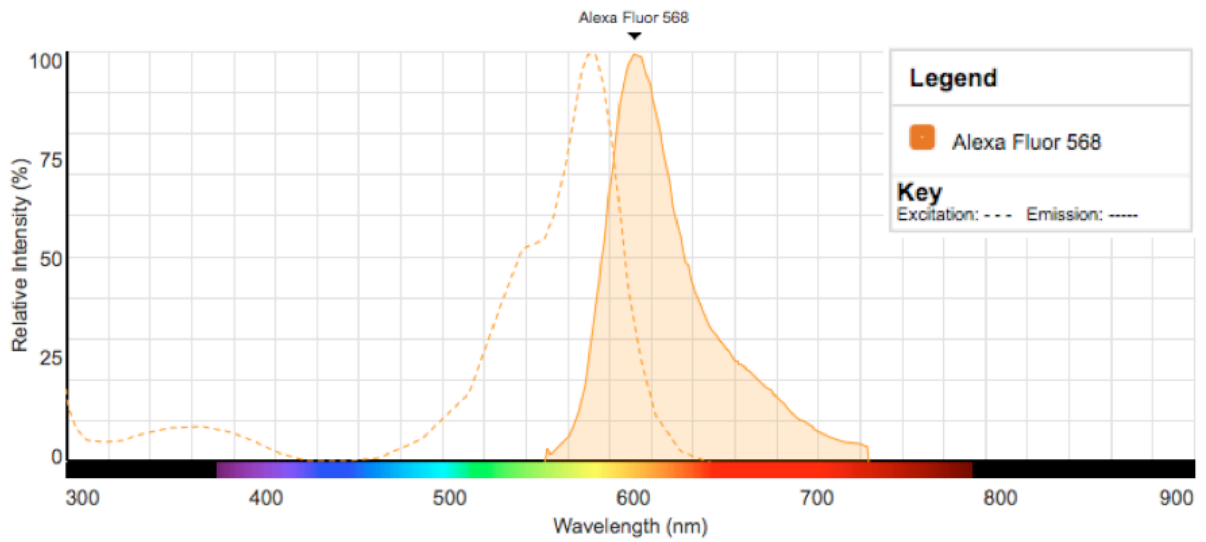
Burlingame, CA, USA), a coverslip was placed over the slides in order to prevent photobleaching.

### 3.6 Data Analysis

For each experimental animal, GAD67 protein expression from 5 coronal sections was measured and compared to sham and control animals. Specifically, 12 sample fields were analyzed in layer IV of the cortex, giving a total measurement of 384, 000  $\mu\text{m}^2$  per section [Figure 3.3]. The sample fields were located across cortical areas, including the predominately visual area (dorsal V2L), audiovisual area (V2L), and the predominately auditory area (dorsal auditory, Au1/AuD) [Figure 3]. Slides were placed on the stage of an Axioplan 2 imaging light microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) under 63 $\times$  magnification. All images were captured with an AxioCam camera (Carl Zeiss Microscopy GmbH, Jena, Germany) and Axiovision Release 4.3 software (Carl Zeiss Microscopy GmbH, Jena, Germany). The 568 nm laser line was used to excite Alexa Fluor 568, which has an emission peak of 603 nm [Figure 3.4]. The exposure time was set to 13 milliseconds to attain a high quality image, while limiting saturation of the pixels. GAD67 protein expression was determined by calculating the fluorescence intensity of GAD67-immunolabelled cells. The expression of GAD67 within the various cortical regions (dorsal V2L, V2L, and dorsal auditory, Au1/AuD) was analyzed by using the data from ten separate images (63 $\times$  magnification), for each animal, in each of area. In each image, GAD67 fluorescence intensity was calculated using ImageJ software. The mean intensity value was collected in each region of interest and background noise was subtracted to reveal the corrected total cell fluorescence (Waters, 2009).



**Figure 3.3.** A coronal section of the rat cortex adapted from Paxinos and Watson 6<sup>th</sup> Edition at bregma -5.50 mm. This image shows the location of the twelve sample fields from which measurements were taken across the predominately visual area (dorsal V2L), audiovisual area (V2L), and the predominately auditory area (dorsal auditory, Au1/AuD). Each field is approximately 32,000  $\mu\text{m}^2$ . Within each field, smaller areas (10,000  $\mu\text{m}^2$ ) were used for analysis of GAD67-immunolabelled cells. In each coronal section, consistency in location was maintained by using the rhinal fissure as an internal landmark.



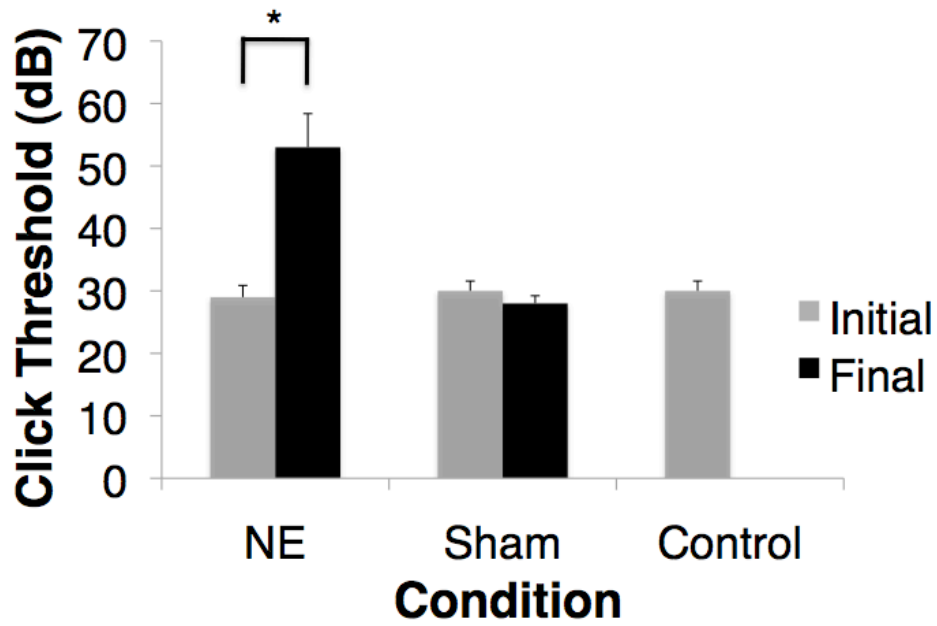
**Figure 3.4.** Fluorescence excitation/emission spectra of Alexa Fluor 568. Alexa Fluor 568 is maximally excited at 578 nm (dashed line) and reaches its emission peak at 603 nm (solid orange). Image courtesy of Fluorescence Spectra Viewer, Life Technologies, 2014.



## 4 Results

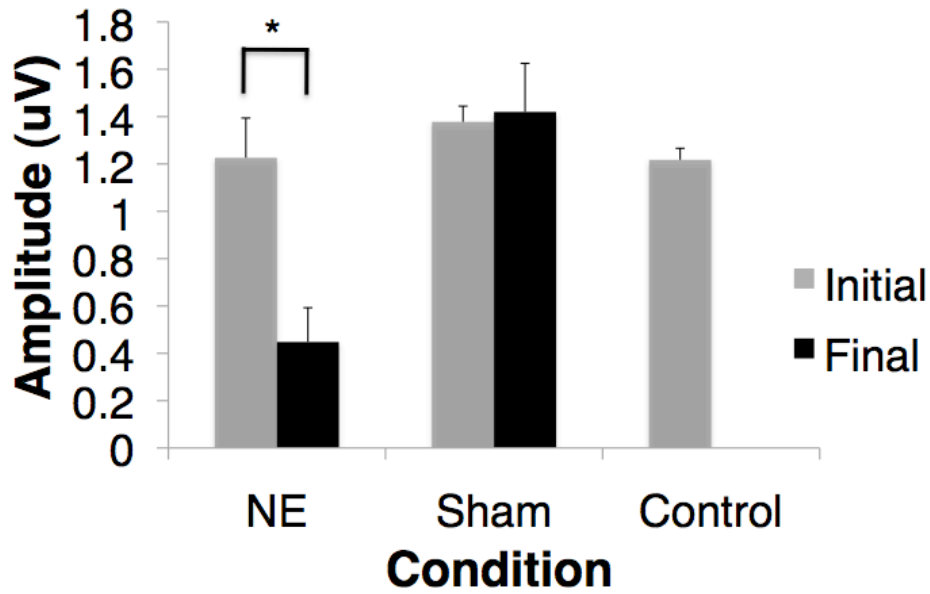
### 4.1 Auditory Brainstem Response Following Noise Exposure

To assess the magnitude of hearing loss following noise exposure, an auditory brainstem response (ABR) was used. All animals ( $n=15$ ) were subjected to an ABR on Day 1 to measure baseline hearing levels. On Day 14, noise-exposed ( $n=5$ ) and sham ( $n=5$ ) animals received a final ABR and the outcomes were compared to baseline levels to provide a functional assessment of the damage caused by noise exposure. A one-way ANOVA was used to confirm there was no significant difference between initial click thresholds across all groups ( $F(2,12)=0.118, p=0.890$ ) [Fig. 4.1]. A mixed repeated measures ANOVA was conducted to determine if initial and final click thresholds in noise-exposed and sham animals varied significantly. There was a significant main effect of time ( $F(1,8)=11.388, p=0.010$ ) and an interaction between time and condition ( $F(1,8)=15.906, p=0.004$ ). Further analysis using a paired t-test indicated that final click thresholds for noise-exposed animals were significantly higher than the initial thresholds ( $t(4)=-3.748, p=0.020$ ) as measured with a 97.5% confidence interval [Fig. 4.1]. The average threshold increase in noise-exposed animals was  $24\pm 3.5$  dB. Initial and final click thresholds for shams did not differ significantly ( $t(4)=1.633, p=0.178$ ).



**Figure 4.1.** Auditory brainstem responses measured in noise-exposed (NE), sham and control animals. Noise exposure caused a significant increase in click thresholds ( $p = 0.020$ ). Error bars represent standard error of the mean.

To further investigate the damage caused by overexposure to high intensity noise, ABR wave I amplitude was examined. A decrease in wave I amplitude following noise exposure is indicative of cochlear damage, even in the absence of a threshold elevation (Kujawa and Liberman, 2009). A one-way ANOVA was used to confirm there was no significant difference in initial wave I amplitudes in noise-exposed, sham and control animals ( $F(2,12)=0.551, p=0.590$ ) [Fig. 4.2]. A mixed repeated measures ANOVA was conducted to determine if initial and final wave I amplitudes in noise-exposed and sham animals varied significantly. There was a significant main effect of time ( $F(1,8)=6.654, p=0.033$ ) and an interaction between time and condition ( $F(1,8)=5.194, p=0.052$ ). Further analysis using a paired t-test indicated that final wave I amplitudes for noise-exposed animals were significantly lower than the initial amplitudes ( $t(4)=3.906, p=0.017$ ) as measured with a 97.5% confidence interval [Fig. 4.2]. The average decrease in wave I amplitude in noise-exposed animals was  $0.78\pm 0.20 \mu\text{V}$ . Initial and final wave I amplitudes for shams did not differ significantly ( $t(4)=0.191, p=0.858$ ).



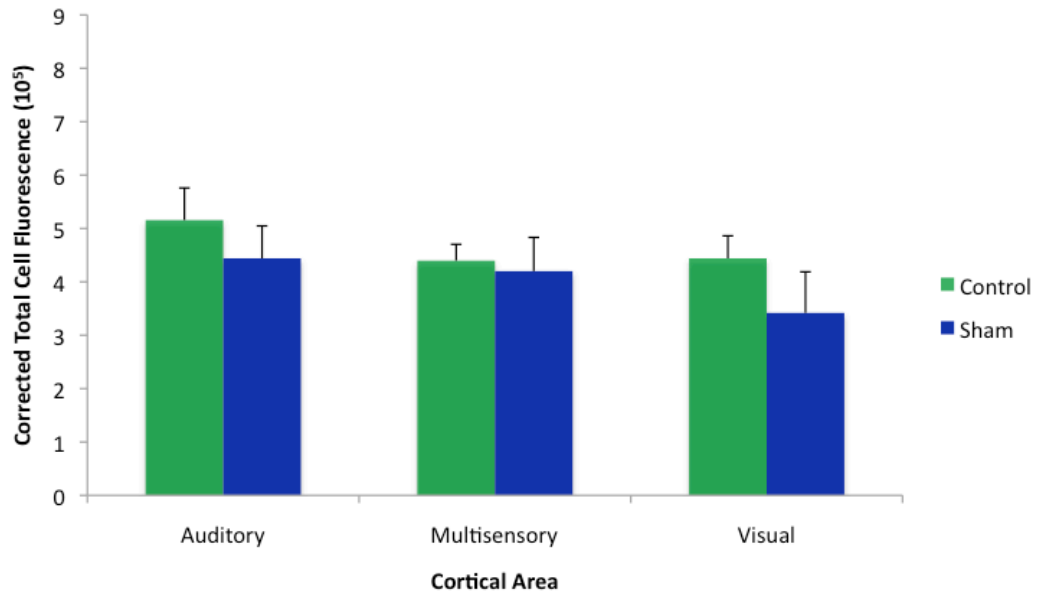
**Figure 4.2.** Auditory brainstem responses measured in noise-exposed (NE), sham and control animals. Noise exposure caused a significant decrease in wave I amplitudes ( $p = 0.017$ ). Error bars represent standard error of the mean.

## 4.2 GAD67 Protein Levels Following Noise Exposure

GAD67 was used as a specific marker for GABA, the major inhibitory neurotransmitter. Specifically, 40  $\mu\text{m}$  coronal sections were collected in serial from bregma -4.5 to -6.5 mm and stained for GAD67 protein levels using immunohistochemical techniques (see materials and methods for details). Layer IV of the cortex was then analyzed in noise-exposed ( $n=5$ ), sham ( $n=5$ ) and control ( $n=5$ ) animals.

### 4.2.1 GAD67 Protein Levels Across Cortical Areas in Sham and Control Animals

To determine if there was a difference in GAD67 protein levels between sham and control animals, a mixed repeated measures ANOVA was conducted. GAD67 protein levels in the auditory (boxes 1 and 2), multisensory (boxes 7 and 8), and extrastriate visual (boxes 11 and 12) cortices were averaged across all coronal sections (bregma -4.5, -5.0, -5.5, -6.0, and -6.5 mm) to achieve a total GAD67 protein level in each area [Figure 4.3]. There was a significant main effect of area ( $F(2,16)=4.619, p=0.026$ ), but no interaction between area and condition ( $F(2,16)=1.058, p=0.370$ ). Post-hoc tests using a Bonferroni correction revealed no significant difference between control and sham animals in any area. Therefore, for the remaining discussion control animals will be disregarded.

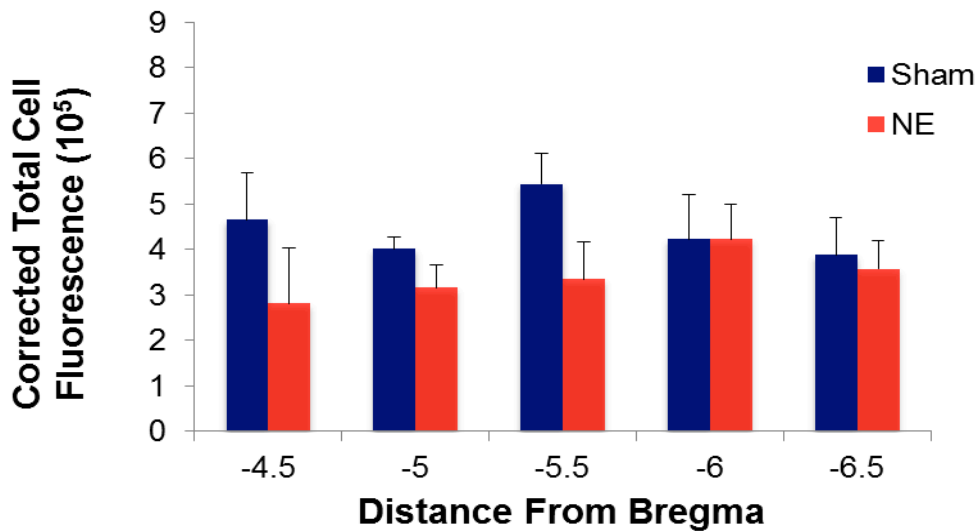


**Figure 4.3.** GAD67-immunofluorescence in the auditory, multisensory, and extrastriate visual cortices in control and sham animals. The conditions were not significantly different. Error bars represent standard error of the mean.

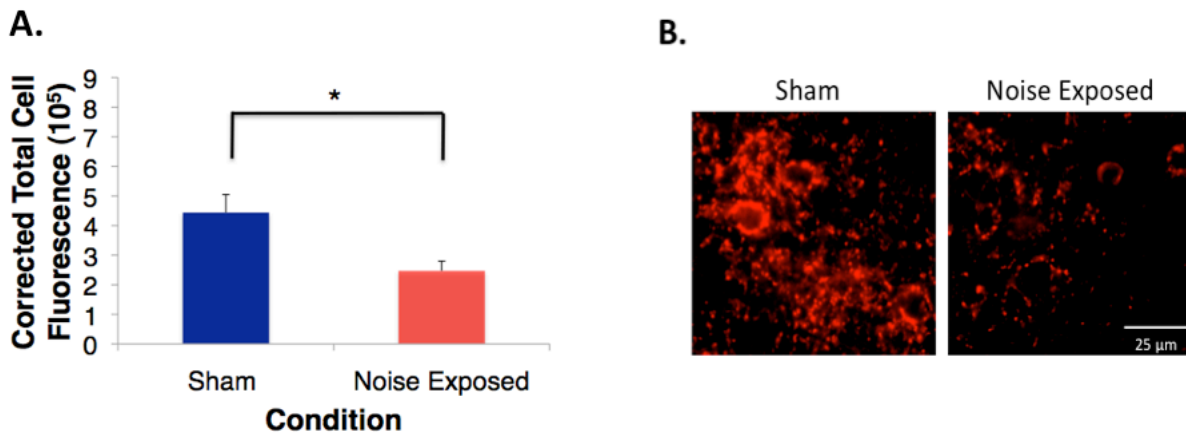
## 4.2.2 Changes in GAD67 Protein Levels in the Auditory Cortex

To examine GAD67 protein levels in the primary auditory cortex of noise-exposed animals, boxes 1 and 2 in each coronal section, from bregma -4.5 to -6.5 mm, were analyzed and compared to shams. Analysis using a mixed repeated measures ANOVA indicated that there was no significant main effect of distance from bregma ( $F(4,32)=1.017, p=0.413$ ) and no interaction between distance from bregma and condition ( $F(4,32)=1.719, p=0.170$ ) [Figure 4.4]. However, there is a trend showing a greater difference in GAD67 protein levels between noise-exposed and sham animals in sections -4.5 to -5.5 bregma, which are the higher frequency auditory regions [Figure 4.4].

When collapsing across distance from bregma, an unpaired t-test demonstrated that there is a significant decrease (44% reduction) in GAD67 protein levels in the primary auditory cortex of noise-exposed animals compared to shams ( $t(8)=2.857, p=0.021$ ) [Figure. 4.5].



**Figure 4.4.** GAD67-immunofluorescence in the primary auditory cortex of sham and noise-exposed animals from bregma -4.5 to -6.5 mm. There was no significant main effect of distance from bregma ( $p=0.413$ ) or interaction between distance from bregma and condition ( $p=0.170$ ). Error bars represent standard error of the mean.



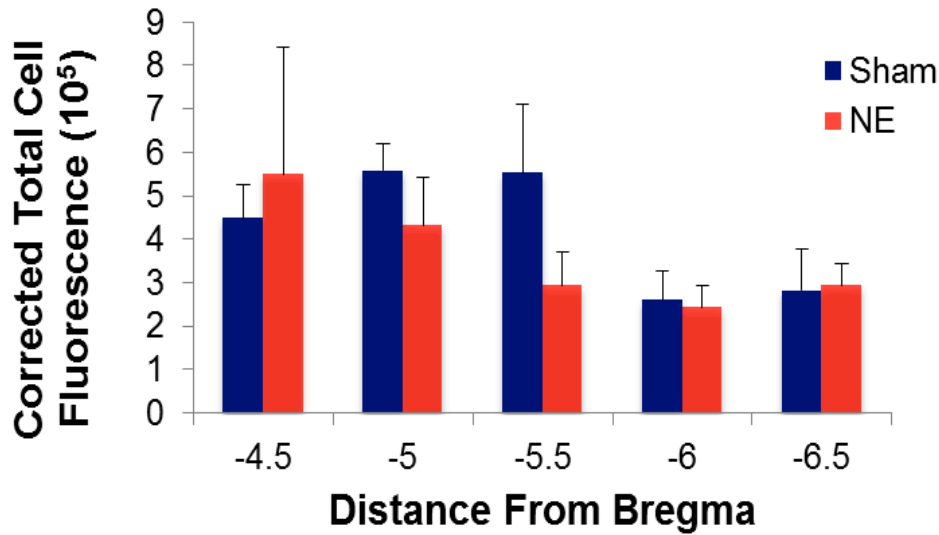
**Figure 4.5.** GAD67-immunofluorescence in the primary auditory cortex of sham and noise-exposed animals. Noise-exposed animals experienced a significant decrease in GAD67 levels ( $p=0.021$ ). Error bars represent standard error of the mean.



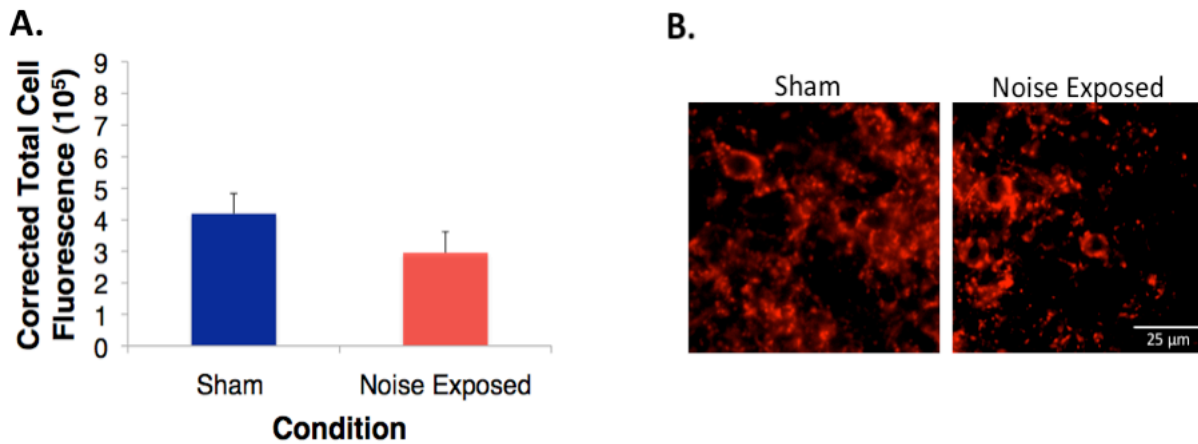
### 4.2.3 Changes in GAD67 Protein Levels in the Multisensory Cortex

To determine GAD67 protein levels in the multisensory cortex of noise-exposed and sham animals, boxes 7 and 8 were examined from bregma -4.5 to -6.5 mm. A mixed repeated measures ANOVA revealed no significant main effect of distance from bregma ( $F(4,32)=1.784$ ,  $p=0.201$ ) and no interaction between distance from bregma and condition ( $F(4,32)=0.637$ ,  $p=0.537$ ) [Figure 4.6]. However, the largest difference in GAD67 protein levels was at bregma -5.5 mm. This result coincides with recent electrophysiological findings in the Allman lab.

Upon collapsing across distance from bregma, an unpaired t-test showed no significant difference in GAD67 protein levels in the multisensory cortex of noise-exposed and sham animals ( $t(8)=1.359$ ,  $p=0.211$ ) [Figure 4.7]. However, there was a trend indicating a 30% reduction in GAD67 protein levels in noise-exposed animals when compared to shams.



**Figure 4.6.** GAD67-immunofluorescence in the multisensory cortex of sham and noise-exposed animals from bregma -4.5 to -6.5 mm. There was no significant main effect of distance from bregma ( $p=0.201$ ) or interaction between distance from bregma and condition ( $p=0.537$ ). Error bars represent standard error of the mean.

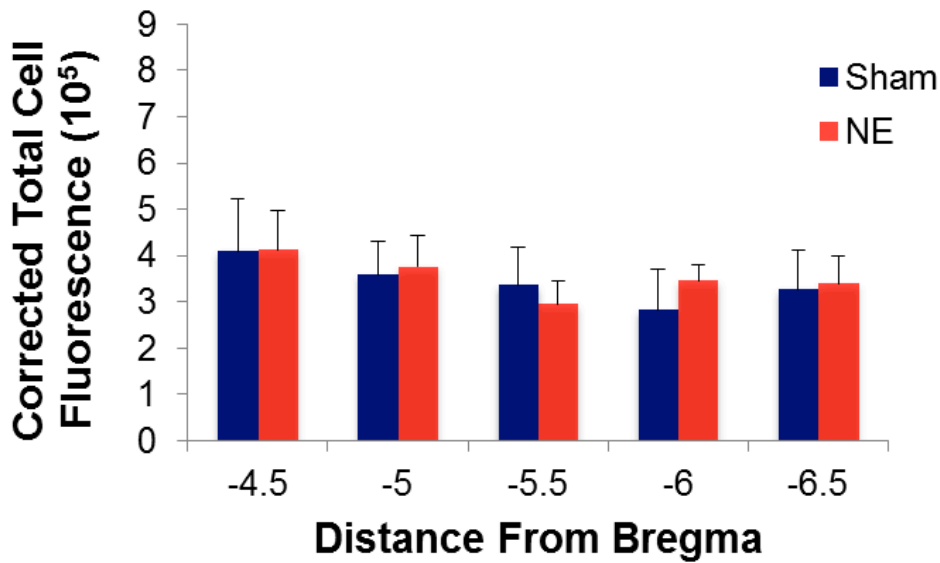


**Figure 4.7.** GAD67-immunofluorescence in the multisensory cortex of sham and noise-exposed animals. There was no significant difference in GAD67 protein levels between conditions ( $p=0.211$ ). Error bars represent standard error of the mean.

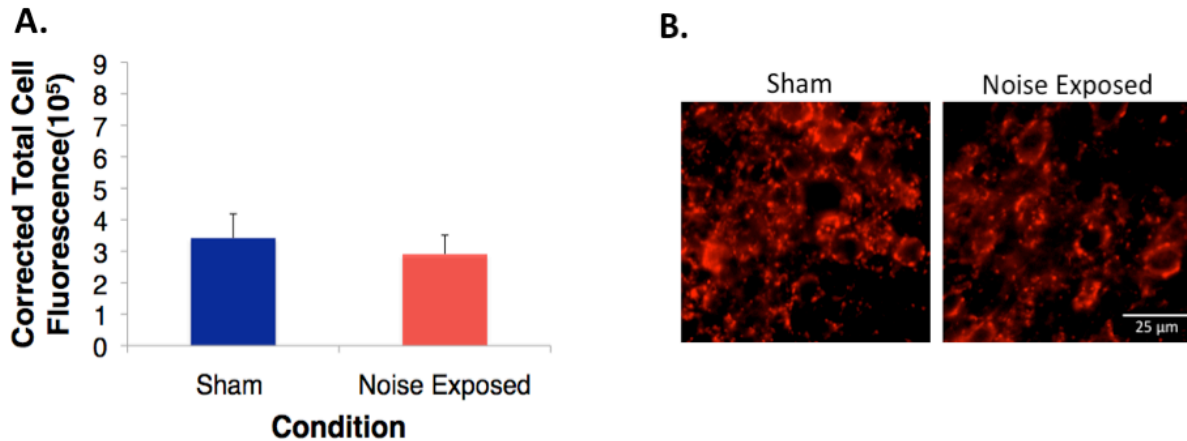
## 4.2.4 Changes in GAD67 Protein Levels in the Extrastriate Visual Cortex

In order to examine GAD67 protein levels in the extrastriate visual cortex, boxes 11 and 12 were analyzed in both noise-exposed and sham animals. To confirm that GAD67 protein levels do not vary throughout the extrastriate visual cortex, coronal sections from bregma -4.5 to -6.5 mm were examined separately. A mixed repeated measures ANOVA illustrated that there was no significant main effect of distance from bregma ( $F(4,32)=1.390, p=0.260$ ) and no interaction between distance from bregma and condition ( $F(4,32)=0.291, p=0.882$ ) [Figure 4.8].

When collapsing across distance from bregma, an unpaired t-test revealed no significant difference in GAD67 protein levels of noise-exposed and sham animals ( $t(8)=0.522, p=0.616$ ) [Figure 4.9]. However, there was a trend showing a 15% reduction in GAD67 protein levels in noise-exposed animals when compared to shams.



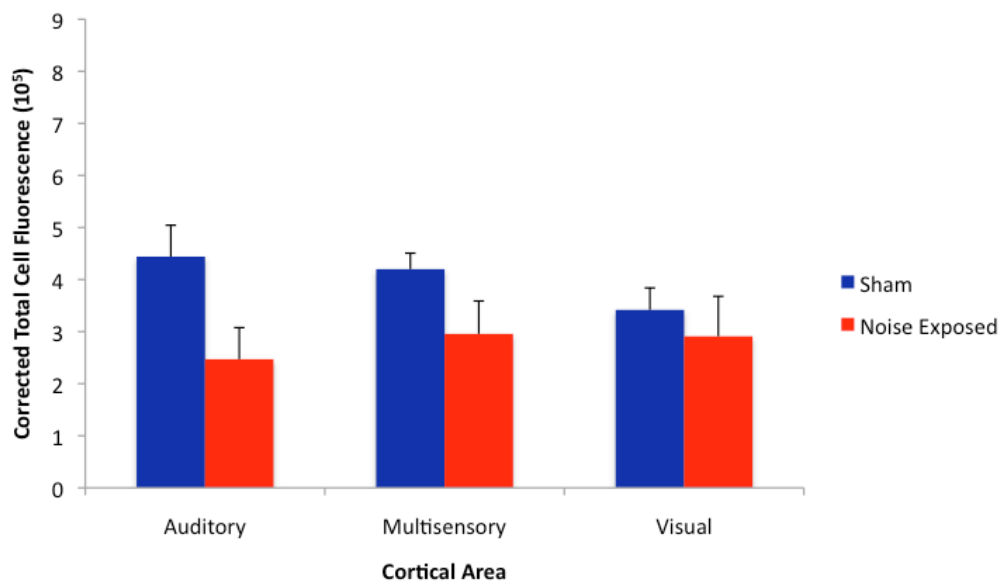
**Figure 4.8.** GAD67-immunofluorescence in the extrastriate visual cortex of sham and noise-exposed animals from bregma -4.5 to -6.5 mm. There was no significant main effect of distance from bregma ( $p=0.260$ ) or interaction between distance from bregma and condition ( $p=0.882$ ). Error bars represent standard error of the mean.



**Figure 4.9.** GAD67-immunofluorescence in the extrastriate visual cortex of sham and noise-exposed animals. There was no significant difference in GAD67 protein levels between conditions ( $p=0.616$ ). Error bars represent standard error of the mean.

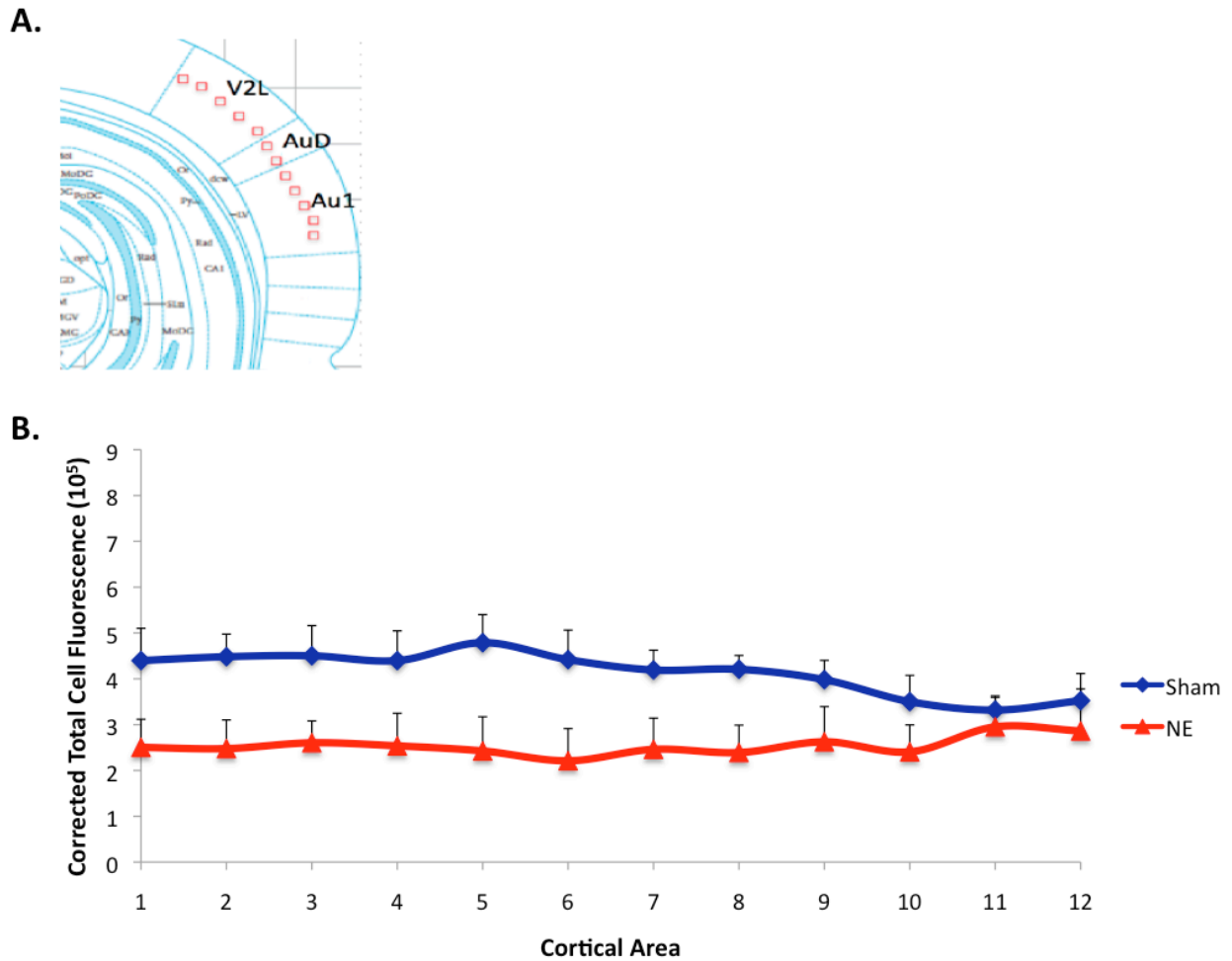
## 4.2.5 Changes in GAD67 Protein Levels Across Cortical Areas

To summarize the differences in GAD67 protein levels between sham and noise-exposed animals, a mixed repeated measures ANOVA was conducted. GAD67 protein levels in the auditory (boxes 1 and 2), multisensory (boxes 7 and 8), and extrastriate visual (boxes 11 and 12) cortices were averaged across all coronal sections (bregma -4.5, -5.0, -5.5, -6.0, and -6.5 mm) to achieve a total GAD67 protein level in each area [Figure 4.10]. There was no significant main effect of area ( $F(2,16)=0.953, p=0.406$ ) and no interaction between area and condition ( $F(2,16)=2.808, p=0.090$ ). However, there is a trend showing a greater difference in GAD67 protein levels in the auditory cortex (44% reduction), as compared to the multisensory (30% reduction) and extrastriate visual (15% reduction) cortices [Figure 4.10].



**Figure 4.10.** GAD67-immunofluorescence across the auditory, multisensory, and extrastriate visual cortices in sham and noise-exposed animals. The conditions were not significantly different. Error bars represent standard error of the mean.

To confirm that there was a gradual change in GAD67 protein levels along the cortex, boxes 1 through 12 were averaged across all coronal sections (bregma -4.5, -5.0, -5.5, -6.0, and -6.5 mm) and analyzed to achieve a total GAD67 protein level for each specific area [Figure 4.11]. A mixed repeated measures ANOVA revealed no significant main effect of area ( $F(11,88)=0.818$ ,  $p=0.622$ ) and no significant interaction ( $F(11,88)=2.219$ ,  $p=0.096$ ). However, there is a trend indicating a greater difference in GAD67 protein levels in the auditory area (boxes 1 and 2) compared to the extrastriate visual area (boxes 11 and 12).



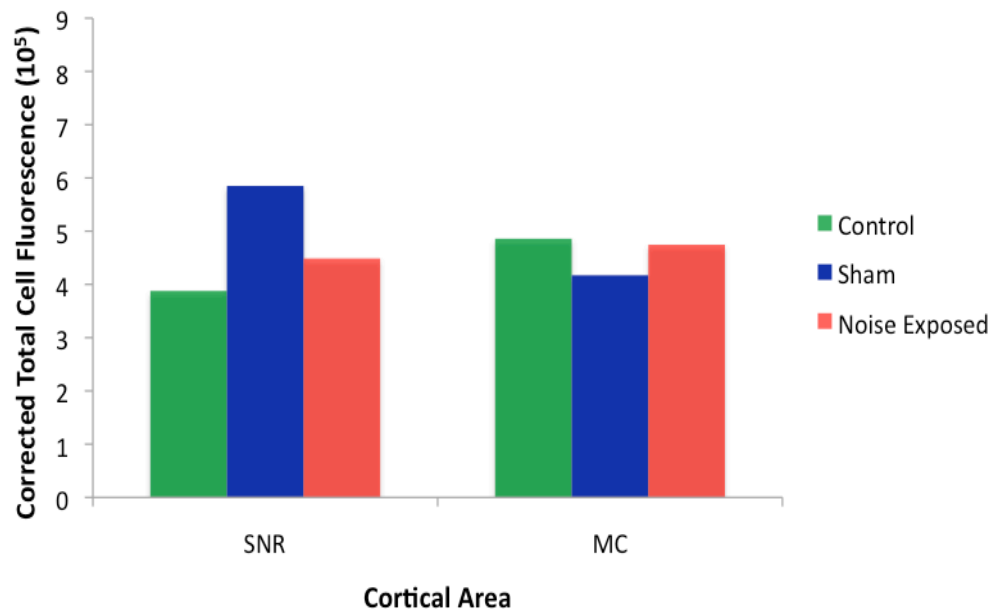
**Figure 4.11.** (A) Schematic indicating areas 1 to 12 moving in a rostral direction along the cortex. Image adapted from Paxinos and Watson, *The Rat Brain Atlas* 6<sup>th</sup> edition. (B) GAD67-immunofluorescence corresponding to boxes 1 through 12, collapsed across all coronal sections (bregma -4.5, -5.0, -5.5, -6.0, and -6.5 mm) in sham and noise-exposed animals. Error bars represent standard error of the mean.

## 4.2.6. Negative Controls

To confirm that the immunohistochemistry results were not affected by parameters in the laboratory, GAD67 levels were also measured in the substantia nigra reticulata, an area not altered by noise exposure. Given that this analysis was only performed in six animals (two from each condition), statistical tests could not be run. However, when looking at the data, there does not appear to be a large difference in GAD67 protein levels in the substantia nigra reticulata between conditions [Figure 4.12].

In addition, the motor cortex, another area that is not affected by noise exposure, was used as a negative control to confirm that there was no difference in GAD67 protein levels in the cortex of animals from each condition [Figure 4.13]. Again, this analysis was not performed on enough animals to run statistical tests, however, the GAD67 protein levels were similar across all groups in the motor cortex.





**Figure 4.12.** GAD67-immunofluorescence in the substantia nigra reticulata (SNR) and motor cortex (MC) in control (n=2), sham (n=2) and noise-exposed (n=2) animals.

## 5 Discussion

### 5.1 Auditory Brainstem Response Following Noise Exposure

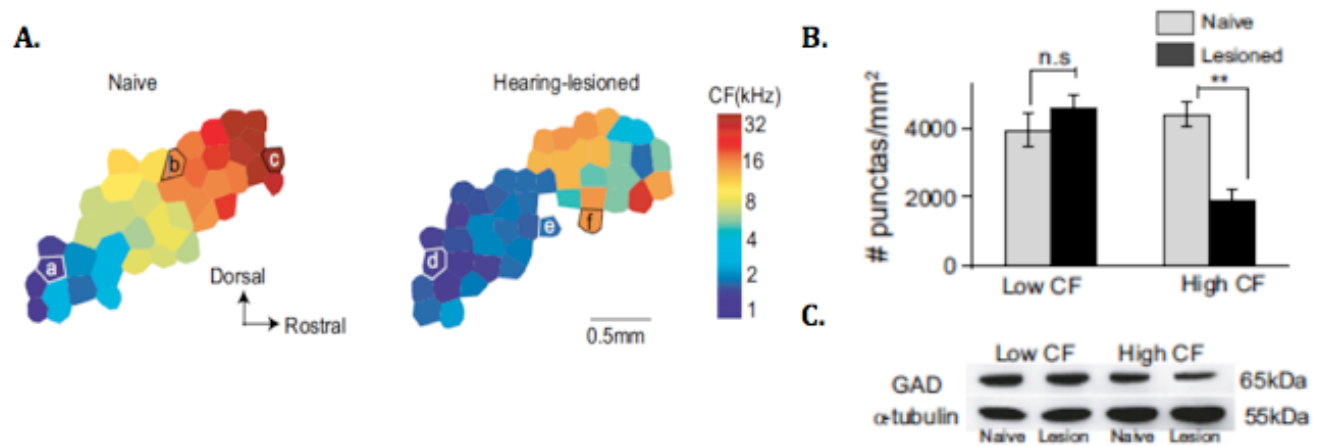
Adult male Sprague-Dawley rats were subjected to a broadband noise (0.8 – 20 kHz) at 120 dB for 2 hours. To assess the damage caused by exposure to high-intensity noise, an auditory brainstem response was performed before and fourteen days following the noise exposure. Both click thresholds and wave I amplitudes were measured. As expected, noise-exposed animals showed a significant increase in click threshold ( $24\pm 3.5$  dB) and decrease in wave I amplitude ( $0.78\pm 0.20$   $\mu$ V), indicating a partial hearing loss. Therefore, the auditory brainstem response confirmed the effectiveness of this previously established noise-exposure protocol.

### 5.2 Changes in GAD67 Protein Levels in the Auditory Cortex

Based on the wealth of academic studies using electrophysiology, targeted lesioning, western blots and/or immunoblots, GABA neurotransmission within the primary auditory cortex has been shown to decrease following acoustic trauma (Xu et al., 2009, Yang et al., 2011). The present study extends the findings of cortical plasticity following hearing loss in adult animals using immunohistochemical techniques to label GAD67 proteins across the auditory, multisensory and extrastriate visual cortices. Given that there was no significant difference in the auditory, multisensory, or extrastriate visual areas between sham and control animals, for the remaining discussion control animals will be disregarded.

Adult male Sprague-Dawley rats with partial hearing loss ( $24 \pm 3.5$  dB threshold) revealed a significant decrease (44% reduction) in the amount of GAD67 protein levels in the primary auditory cortex compared to shams. Furthermore, when examining individual coronal sections from bregma -4.5 to -6.5 mm there was a trend indicating a lesser amount of GAD proteins in sections -4.5, -5.0 and -5.5 mm as compared to sections -6.0 and -6.5 in noise-exposed animals. This uneven distribution is likely a result of the tonotopic organization in both the peripheral and central auditory systems. In the peripheral auditory system, the cochlea is ordered such that different frequencies are transmitted separately along specific parts of the basilar membrane (Auditory System: Structure and Function, 1997). At the base of the cochlea only high frequency vibrations move the membrane and thus the hair cells, however, as one travels towards the apex lower frequencies are able to move the hair cells. This tonotopic organization remains linear in relation to its position in the cochlea as it projects to the primary auditory cortex. There is an orderly representation of spectral frequencies throughout the primary auditory cortex (Eggermont and Roberts, 2004), such that higher frequency sounds are decoded rostrally, and lower frequencies more caudally. Therefore, the location of damage induced by acoustic trauma depends on the frequency composition of the stimulus (Slepecky, 1986). If an individual is subjected to a high frequency noise exposure, hair cells at the base of the cochlea will experience the greatest amount of damage (Bohne, et al., 1987). Furthermore, Yang et al. (2011) determined that high-frequency hearing loss also results in a sensory-deprived cortical region with decreased inhibitory synaptic transmission rostrally, and a neighboring normal hearing region (caudally) with map reorganization [Figure 5.1]. Additionally, given the spatial arrangement of the cochlea, both high and low frequency sounds must travel through the base of the cochlea (high frequency region) to reach their specific destinations. Thus, exposure to

broadband noise, which affects a wide range of the cochlea, should cause greater damage to higher frequency regions compared to lower frequency regions and thus a larger degree of plasticity to rostral areas of the cortex. This coincides with our immunohistochemical findings in the primary auditory cortex.



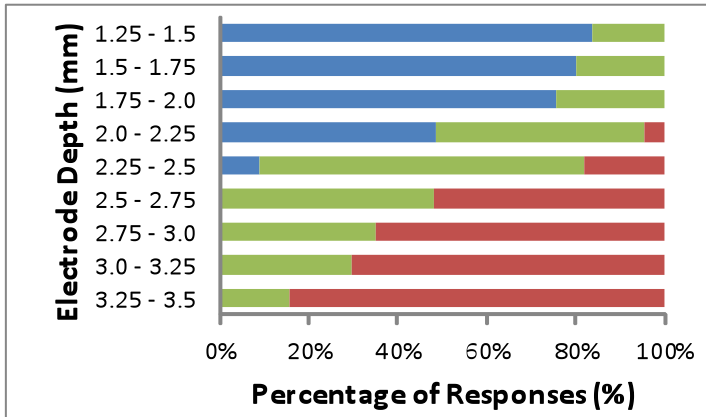
**Figure 5.1.** High frequency hearing loss caused cortical map reorganization with decreased inhibitory synaptic transmission. (A) Cortical maps and receptive fields in naïve and hearing-lesioned animals. (B) Reduced density of GAD65 puncta in the high characteristic frequency (CF) zone of hearing-lesioned animals. (C) Western blot revealed decreased GAD65 expression in high-CF areas of hearing-lesioned animals. \*\*  $p < 0.01$ . Image adapted from Yang et al. (2011).

## 5.3 Changes in GAD67 Protein Levels in the Multisensory Cortex

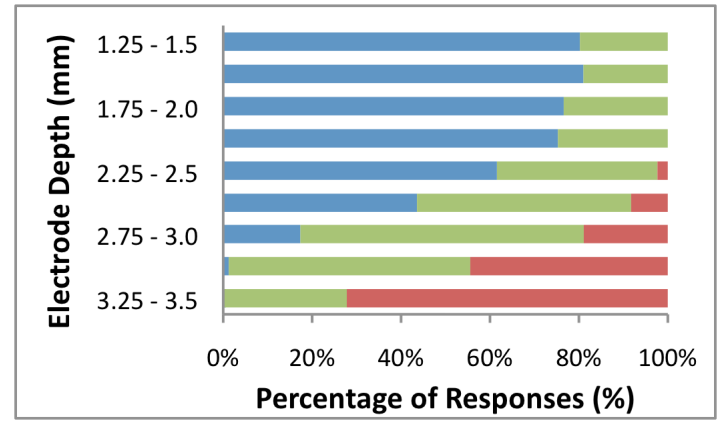
When examining individual cortical layers, from bregma -4.5 to -6.5 mm, we saw the greatest difference in GAD67 protein levels in the multisensory cortex between sham and noise-exposed animals at bregma -5.5 mm. This result corresponds with electrophysiological findings in the Allman laboratory, which demonstrated that the largest increase in the proportion of visually responsive neurons in the multisensory cortex between control and noise-exposed animals occurred at bregma -5.5 mm.

Noise-induced partial hearing loss did not cause a significant decrease ( $p=0.211$ ) in the amount of GAD67 protein levels in the multisensory cortex compared to shams. There was, however, a 30% reduction of GAD levels in noise-exposed animals. This finding agrees with recent extracellular electrophysiological recordings in our laboratory that revealed an increase in the proportion of neurons responsive to visual stimuli in the primary auditory and neighboring multisensory cortical regions following the same high intensity noise exposure used in the current experiment. This electrophysiological work established that noise-induced partial hearing loss leads to cortical plasticity that extends beyond the primary auditory cortex into nearby areas that normally integrate audio-visual information. In addition, this confirms the validity of the noise exposure we used, as it has been shown to result in cortical plasticity.

## Control



## Noise Exposed



**Figure 5.2.** Extracellular electrophysiological recordings revealed an increase in the number of neurons responsive to visual stimuli in auditory and multisensory cortices of noise-exposed animals. Visually responsive neurons (blue), multisensory neurons (green) and auditory neurons (red). (Personal communication with A. Schormans, March 2015).

## 5.4 Changes in GAD67 Protein Levels in the Extrastriate Visual Cortex

As anticipated, there was a relatively even distribution of GAD across the coronal sections (i.e. distance from bregma) in both noise-exposed and sham animals. Furthermore, high-intensity noise exposure did not cause a significant decrease ( $p=0.616$ ) in GAD67 protein levels in the extrastriate visual cortex compared to shams.

## 5.5 Changes in GAD67 Protein Levels Across Cortical Areas

To confirm that there was a gradual change in GAD67 protein levels from rostral to caudal along the cortex, boxes 1 through 12 were averaged across all coronal sections (bregma -4.5, -5.0, -5.5, -6.0, and -6.5 mm) and analyzed to achieve a total GAD67 protein level for each specific area. There was a trend indicating that the difference in GAD67 levels between noise-exposed and sham animals was greatest in the primary auditory area (boxes 1 & 2) and gradually decreased along the cortex, towards the extrastriate visual area (boxes 11 & 12) where the smallest difference in GAD levels was found. This analysis was used to confirm that GAD levels did not change drastically in the areas between the primary auditory, multisensory, and extrastriate visual cortices.



## 5.6 Negative Controls

Given that parameters in the laboratory (such as temperature, exact time in solutions and washes, etc.) can affect the accuracy of immunohistochemical staining, GAD67 levels were also measured in the substantia nigra reticulata, an area not altered by noise exposure. Since we did not see a large difference in GAD levels in this area between conditions, we can rule out the possibility of daily parameters having an impact on our results.

In addition, we wanted to confirm that there was no difference in GAD67 protein levels in the cortex of animals from each condition. To control for this, GAD levels were measured in the motor cortex of animals from each condition (motor cortex is also not affected by noise exposure). We did not see a large difference in GAD levels between conditions when investigating the motor cortex.

## 5.7 Significance of the Study

Firstly, this study is important because it uses immunohistochemical staining as an alternate method to validate our current understanding of noise-induced cortical plasticity. It is interesting to see our immunohistochemistry and histology results build upon previously established extracellular electrophysiological recordings, as well as determine the impact of noise-induced hearing loss on inhibitory neurotransmission in various areas of the cortex. Secondly, it is important to look at the big picture and study noise-induced hearing loss and its cortical modulations for its clinical relevance. Hearing loss is one of the most prevalent auditory impairments and it occurs when there is reduced sensitivity to the sounds normally heard. This

disorder is among the most common medical conditions, affecting ten percent of the population (Oishi and Schacht, 2011). Noise exposure, along with genetics and aging are the major causes of this disorder, with noise exposure leading to approximately half of all cases of hearing loss (Oishi and Schacht, 2011). Therefore, a better understanding of the molecular mechanisms involved in noise-induced hearing loss will help to determine potential drug targets and possible therapies for affected individuals.

## 5.8 Limitations

As with any scientific study, there are a few limitations that need to be acknowledged. When performing the negative controls, data was not collected from enough animals to run statistical tests in order to confirm there was no significant difference in GAD levels between conditions. Additionally, there is inherent bias involved when using immunohistochemical techniques. In order to avoid this issue, a new stereotaxic microscope has recently become available. This stereotaxic microscope allows one to outline their areas of interest on individual sections, and then it will take randomized images within each specified area. This new technique rules out any bias associated with image/data collection.

## 5.9 Concluding Remarks

As expected, we observed a significant reduction in GAD67 protein levels in the primary auditory cortex of noise-exposed animals compared to shams ( $p=0.020$ ). Consistent with our hypothesis, noise-induced hearing loss caused a differential effect on GAD67 levels in the various cortical areas; the primary auditory cortex showed the greatest reduction (44%) between sham and noise-exposed rats, compared to the multisensory (30% reduction) and extrastriate visual (15% reduction) cortices.

## 6 References

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## APPENDIX A

### Curriculum Vitae

<b>Name:</b>	Sarah Fitzpatrick
<b>Post-secondary Education and Degrees:</b>	Western University London, Ontario, Canada 2009-2013 BSc. Honours Specialization
<b>Honours and Awards:</b>	Western Graduate Research Scholarship (WGRS) 2013-2015  Anatomy and Cell Biology (ACB) Graduate Entrance Scholarship 2013-2014
<b>Related Work Experience:</b>	Teaching Assistant for Dentistry – Head and Neck Anatomy Western University 2014-2015  Head Teaching Assistant – Human Systemic Anatomy 3319 Western University 2015 Spring  Teaching Assistant for Medicine – Gross Anatomy Western University 2014 Fall  Teaching Assistant for Nursing – Functional Anatomy Western University 2014  Teaching Assistant – Human Systemic Anatomy 3319 Western University 2013-2014  Teaching Assistant for Medical Sciences 4930 Western University 2013 Fall
<b>Publications:</b>	Published abstract for American Association of Anatomists (2015)

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