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Evaluating Changes in Reactive Oxygen Species (ROS) as a Plausible Mechanism Underlying the Effect of Noise on the Dopamine System in the Hub for Central Auditory Processes

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EVALUATING CHANGES IN REACTIVE OXYGEN SPECIES (ROS) AS A PLAUSIBLE MECHANISM UNDERLYING THE EFFECT OF NOISE ON THE DOPAMINE SYSTEM IN THE HUB FOR CENTRAL AUDITORY PROCESSES

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has been approved as meeting the requirements for the Degree of Master of Science in the Department of Chemistry and Biochemistry in the College of Natural and Health Sciences.

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ABSTRACT

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Excessive exposure to noise leads to hearing loss but the mechanism by which this happens remains unknown. Presently, dopamine, a neurotransmitter widely known for its involvement in learning and reward-based actions, has also been linked to auditory processes in the inferior colliculus, a principal integration center for hearing and related processes. Preliminary data from our research laboratory, has demonstrated that exposure to deafening noise leads to decreased dopamine release in the inferior colliculus, thus implicating the dopamine system in hearing loss. One plausible mechanism underlying this noise-induced alteration in dopamine neurotransmission could be via excessive production of reactive oxygen species (ROS). Reactive oxygen species can also modulate synaptic transmission. Thus, we hypothesize that loud noise would trigger overproduction of ROS, specifically hydrogen peroxide (H_2O_2) and in turn attenuate dopamine release in the inferior colliculus. Since excessive production of H_2O_2 could result in the depletion of adenosine triphosphate (ATP), we also evaluate the effect of the noise exposure on ATP levels. In order to determine the effect of deafening noise on proteins that regulate dopamine synthesis, we analyze biometals that act as prosthetic groups and may be involved in protein coordination and neurotransmission. The present work utilizes colorimetric assays and XRF analysis to examine changes in H_2O_2, ATP and biometal concentrations of Fe, Ca, K and Zn in the inferior colliculus of adult Sprague Dawley rats following exposure to loud noise. Finally, we explore the possibility of minimizing the effect of noise through antioxidant

(α-lipoic acid) administration (50 mg/kg -I. P). The results of this study demonstrate that loud noise significantly increases the production of H_2O_2 ($p < 0.0001$, n = 10 rats/group). However, the noise exposure had no significant effect on ATP levels ($p = 0.5850$, 10 rats/group). Furthermore, noise exposure caused a significant increase in Fe, Ca and K levels (n = 5 rats/group). The administration of the antioxidant, α-lipoic acid reduced the concentration of H_2O_2 in the noise exposed group ($p < 0.0001$, n = 9 rats/group) and restores biometal concentration for Fe, Ca and K (n = 5 rats/group) to levels comparable with the control but has no effect on ATP. These results expand our understanding on the involvement of dopamine in deafness related changes in the inferior colliculus and open doors to further explore neural mechanisms underlying dopamine's involvement in hearing.

THESIS ACKNOWLEDGEMENT

My eternal gratitude goes to my Heavenly Father who has kept and preserved me throughout my life and through the duration of my master's degree. 'But those who trust in the Lord will find new strength. They will soar high on wings like eagles. They will run and not grow weary. They will walk and not faint' (Isaiah 40:31, New Living Translation).

I would like to thank my research advisor, Dr Apawu for his superior guidance and support throughout my master's degree. I could not have asked for a better supervisor. Besides my advisor, I would like to express my gratitude to my thesis committee members: Dr Zhao and Dr Watzky of the Chemistry Department and Dr James of the Biology Department.

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Last but not the least, I am grateful to my family for all their support. To my husband Kofi Gyamera Akenten Owusu, thank you for being the most supportive, understanding husband. To my daughter, Maria Ohemaa Ago Owusu, you are a ray of sunshine in my life. Your presence in my life reminds me to keep persisting and persevering since I know you will always look up to me as your mother. Finally, my gratitude goes to my parents Evans Doe and Francisca Gasinu for providing me with world class education which has been extremely beneficial in achieving this milestone. I am extremely blessed to have you all as family.

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ATP – Adenosine Triphosphate

GSH – Reduced Glutathione

GSSG – Oxidized Glutathione

H_2O_2 – Hydrogen Peroxide

HRP – Horse Radish Peroxidase

NADPH – Nicotinamide Adenine Dinucleotide Phosphate Reduced

$NADP^+$ - Nicotinamide Adenine Dinucleotide Phosphate Oxidized

NOX2 - NADPH Oxidase 2

PKA - Protein Kinase A

$^{\bullet}OH$ – Hydroxyl radical

O_2^- - Superoxide anion

ROS – Reactive Oxygen Species

SOD - Superoxide Dismutase

TyrH – Tyrosine Hydroxylase

XRF – X-ray Fluorescent Spectrometry

CHAPTER I

INTRODUCTION

Hearing loss is one of the most debilitating physical conditions in the world. According to the World Health Organization (WHO), over 466 million individuals constituting 5% of the world's population suffer from hearing loss (*Deafness and Hearing Loss*, n.d.). WHO predicts that by 2050, 900 million individuals will suffer from disabling hearing loss (*Deafness and Hearing Loss*, n.d.).

Hearing loss may occur as a result of aging or as a side effect of various medications or may be frequently caused by environmental exposures to excessive noise. Industry or factory workers, construction workers, servicemen and musicians are at a high risk of hearing loss or its related conditions including tinnitus due to the noisy nature of their work environments. These individuals may experience full blown hearing loss during their working years or after retirement, leading to a reduced quality of life, and sometimes depression and even suicide. Furthermore, hearing loss causes a loss in productivity of about US$ 750 billion annually worldwide (*Deafness and Hearing Loss*, n.d.).

Unfortunately, the pathophysiology that underlies hearing loss and its related conditions like tinnitus remains complex and poorly understood. Presently, sufficient amount of data has linked noise-induced hearing disorders to neuronal changes in the central auditory nuclei, including the inferior colliculus. The inferior colliculus is part of the auditory brainstem and it is the principal integration center for hearing and related processes. More recently, dopamine has been implicated in auditory processes in the inferior colliculus. Dopamine is a neurotransmitter

known for its involvement in memory, learning and reward-based actions, motor control and cognition. It is widely studied in disorders such as Parkinson's and Alzheimer's diseases, Schizophrenia and addiction. Although, the exact role of dopamine in the inferior colliculus is yet to be defined, available data have shown that loud noise exposure decreases the gene expression for tyrosine hydroxylase (TyrH) (a rate limiting enzyme in the synthesis of dopamine) in the inferior colliculus, implying diminished dopamine levels (Fyk-Kolodziej et al., 2015b). Furthermore, patients with Alzheimer's and Parkinson's diseases have exhibited mild to severe hearing loss, linking dysfunction in the dopamine system to hearing deficit (Segura-Aguilar et al., 2014).

Current data from our research laboratory, measured by Fast scan cyclic voltammetry, have also demonstrated that loud noise leads to decreased dopamine release in the inferior colliculus of adult Sprague Dawley rats (Wilson, n.d., p.50). One plausible mechanism underlying this noise-induced alteration in dopamine neurotransmission could be via excessive production of reactive oxygen species (ROS). Over production of ROS is a major contributor to noise-induced hearing loss (Park et al., 2014). Reactive oxygen species can also modulate neuronal processes, including synaptic dopamine release via regulated export using the calcium (Ca^{2+}) channels pre-synaptically (Chen et al., 2001) and the ATP-sensitive K^+ (K_{ATP}) channels post-synaptically (Rice, 2011). Thus, we hypothesize that loud noise would trigger overproduction of ROS, specifically hydrogen peroxide (H_2O_2) and in turn attenuate dopamine release in the inferior colliculus. Excessive production of H_2O_2 results in the depletion of adenosine triphosphate (ATP) (Chen et al., 2001). Because ATP is the energy currency of the cell, its depletion will cause enzymes that are normally activated through phosphorylation to no longer be activated as will be the case with TyrH (Figure 1).

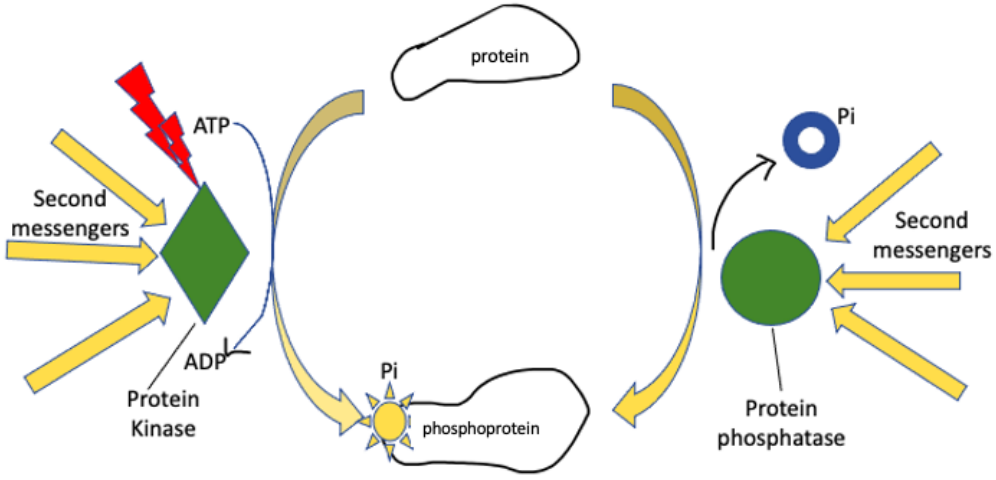


Figure 1: Regulation of cellular proteins by phosphorylation. In the presence of intracellular secondary messengers such as cyclic AMP, there is the activation of Protein kinase which effects the ATP-dependent phosphorylation of proteins such as TyrH. Phosphorylation of TyrH results in its activation hence dopamine is synthesized whiles dephosphorylation results in its deactivation.

Also, in the presence of ROS, there could be oxidative modification of TyrH which is required in the synthesis of dopamine. Oxidative modification occurs through nitration at three tyrosine residues located in a flexible loop close to the enzyme active site (Daubner et al., 2011). The nitration of TyrH results in its inactivation due to a loss of its active conformation. Another oxidative modification which may occur at six of the seven cysteine residues is thiolation (Daubner et al., 2011). This modification also results in the inactivation of TyrH. It is yet to be proven whether these oxidative modifications have any physiological role in noise-induced hearing loss. However, these modifications to an important rate-limiting enzyme in the synthesis of dopamine would lead to dysfunction in the dopamine neurotransmission and related processes.

In the present work, we evaluated the mechanistic options in three main areas. Firstly, we sought to answer whether loud noise increases excessive production of ROS particularly, H_2O_2 in the inferior colliculus of adult rat brain. Secondly, we examined whether the effect of

noise attenuates ATP levels and subsequently diminish dopamine synthesis. Finally, we verified if the noise induced impact on the dopamine system could be prevented through antioxidant (α-lipoic acid) administration prior to noise exposure. This antioxidant was chosen because its racemic mixture has a greater bioavailability and has a longer half-life *in vivo* (Salehi et al., 2019). Reactive oxygen species can be targeted by the administration of antioxidants which have been proposed to prevent or slow noise- induced hearing loss (Tavanai & Mohammadkhani, 2017). Therefore, with the administration of α-lipoic acid, we seek to explore whether the noise-induced alterations in the dopamine system can be abolished or minimized.

Overall, the outcome of this work has the potential to significantly expand our understanding on the role of dopamine in central auditory processes and open doors for therapeutic options that would ensure timely intervention for individuals who suffer or are at high risk of noise-induced hearing loss.

Specific Objectives

O1 Assessing the Impact of Loud Noise on the Production of Reactive Oxygen Species, Specifically Hydrogen Peroxide (H_2O_2) in The Inferior Colliculus.

The central auditory system requires that several major players maintain their modes of action while at the same time achieving integration in order to accomplish the complex sensory action of hearing (Schnupp et al., 2011). This work sought to explore the relationship between dopamine and the central auditory system since the exact role of dopamine in the central auditory system is not known. The expression of TyrH has been reported in the pockets of inferior colliculus, a key region in the central auditory pathway (Holt et al., 2005; Nevue et al., 2016). Recent research data have shown that excessive noise exposure decreases TyrH which will lead to diminished dopamine levels (Fyk-Kolodziej et al., 2015b). Another independent study

demonstrated that excessive noise exposure leads to production of reactive oxygen species (ROS) (Park et al., 2014). Current data from our research laboratory has also demonstrated that loud noise led to decreased dopamine release in the inferior colliculus of adult Sprague Dawley rats (Wilson, n.d., p.50). Thus, we hypothesized that loud noise would trigger production of ROS, specifically H_2O_2 (as previously shown by Park et al., 2014) and could be responsible for the noise induced attenuation of dopamine release observed in the inferior colliculus. To verify this hypothesis, H_2O_2 levels in the inferior colliculus were measured following noise exposure. Per our hypothesis, we expected to see an increase in H_2O_2 production triggered by the noise exposure, which could be interpreted as evidence of oxidative stress.

O2 Examining the Relationship Between Noise Induced Changes in Hydrogen Peroxide Levels and Dopamine Neurotransmission (is the Relationship Mediated by Adenosine Triphosphate (ATP)?)

While the exact role of adenosine triphosphate in neurotransmission is somewhat understood, the underlying mechanisms through which diminished ATP levels may modulate dopamine neurotransmission remain elusive. However, several studies have indicated that ATP may actually have a role in neuromodulation, and these are summarized below.

Dopamine neurotransmission has been shown to be regulated by two channels: the Calcium (Ca^{2+}) channels regulating pre-synaptically (Chen et al., 2001) and the ATP-sensitive K^+ (K_{ATP}) channels that inhibit dopamine neuron firing post-synaptically (Rice, 2011). Both of these channels are regulated by ATP-dependent protein-receptor interactions. Some of these protein-receptor interactions include the D2 channel proteins interacting with G-protein coupled receptors, the dopamine transporter and the dopamine receptors. These protein-receptor interactions are heavily ATP-dependent processes since they require ATP for activation. Furthermore, it has been shown that ATP is a required co-factor in the phosphorylation and

consequent activation of TyrH as shown in Figure 2. On the other hand, it has been shown that endogenous H_2O_2 can activate both Ca^{2+} and K_{ATP} channels (Rice, 2011) and subsequently lead to inhibition of dopamine release. Exogenous levels of H_2O_2 elicit the diversion of Nicotinamide Adenine Dinucleotide Phosphate Reduced (NADPH) to serve as co-factor in the detoxication mechanism involving glutathione peroxidase (Casteilla et al., 2001). By doing this, the presence of high levels of H_2O_2 elicits an ATP-exhaustive process by the diversion of NADPH. All these go to support our hypothesis that H_2O_2 is involved in neuromodulation of dopamine due to the creation of an ATP-exhaustive environment. Figure 2 illustrates this mechanism clearly where inhibition in ATP decreases cyclic adenosine monophosphate (cAMP) signaling, causing deactivation of TyrH and ultimately down regulation in dopamine transmission.

Following noise exposure, ATP was measured using a colorimetric assay developed by Sigma-Aldrich®.As per our hypothesis, we expected a decrease in ATP levels in the noise exposed group as compared to the control group. If so, the decrease would be attributed to the fact that excessive H_2O_2 causes depletion in NADPH which normally will produce approximately three ATP molecules. Therefore, for every molecule of H_2O_2 broken down by glutathione peroxidase, three ATP molecules are used. We expect this process to cause Protein Kinase A (PKA) to be deactivated for the lack of its co-factor ATP. Thus, TyrH activation would be reduced leading to decrease in dopamine synthesis and release (Figure 2).

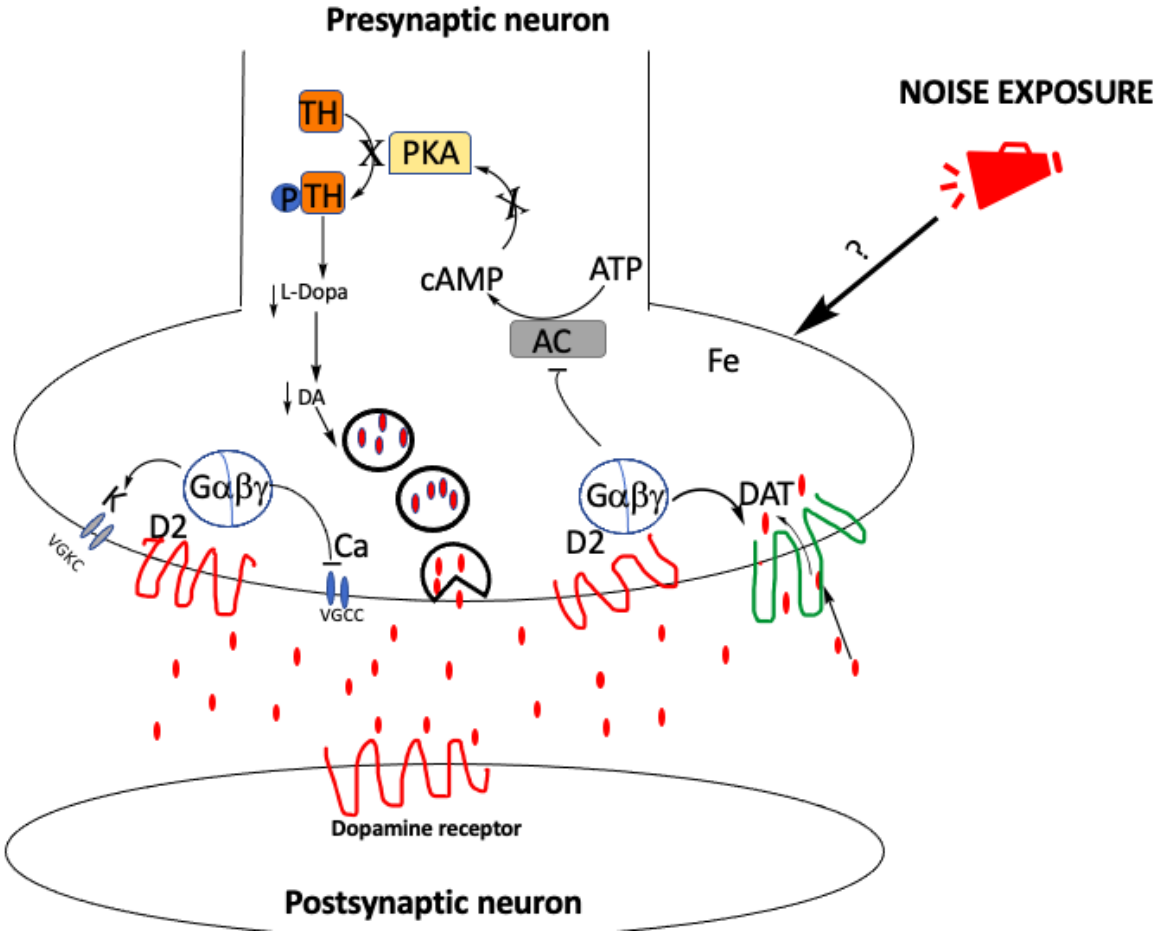


Figure 2: Dopamine neurotransmission. Complex interplay of various proteins and how they interact in a feedback inhibition loop of the dopamine system. Through a cascade of reactions starting downstream, the D2 auto receptors elicit inhibitory effect on a series of reactions including deactivation of TyrH through PKA deactivation and ultimately result in inhibition of dopamine synthesis and release. DAT: Dopamine transporter.

O3 Assessing the Impact of Noise Exposure on the Biometals Involved in Neurotransmission.

As mentioned previously, dopamine neurotransmission has been shown to be regulated by two channels: the Calcium (Ca^{2+}) channels regulating pre-synaptically (Chen et al., 2001) and the ATP-sensitive K^+ (K_{ATP}) channels that inhibit dopamine neuron firing post-synaptically (Rice, 2011). Zinc is an important biometal involved in the coordination of neurological proteins while Fe protects against oxidative damage. Neurodegenerative diseases have been shown to correlate with the shift in trace metal concentration or the disturbance in physiological concentration. Indeed, a 339% increase in Zn, 177% increase in Fe and 4653% increase in Ca have been observed in mouse models of Alzheimer's disease (Leskovjan et al., 2009). Therefore, it is important to investigate the changes that may occur in the concentration of trace metals. This measurement will be done with X-ray fluorescent Spectrometry since it has been shown to be a highly sensitive method for trace metal analysis, detecting concentrations as low as parts per million in biological samples (Grochowski et al., 2019).

Aside from Ca and K which have relatively higher concentrations in the physiological environment, certain elements such as Fe and Zn have been shown to be essential for brain development. Because they are found in less abundance than other elements in the body, they are referred to as trace biometals. Zinc is well recognized as an allosteric modulator of enzyme action and functions as a prosthetic group/catalyst in many enzyme reactions, including superoxide dismutase (Huidobro-Toro et al., 2008). Therefore, it is important that the effect of noise on Zn concentration should be explored as it is the second most abundant trace metal, after Fe, involved in many biological processes (Huidobro-Toro et al., 2008). Ninety percent of Zn in the body is found bound to metalloproteins while the remaining 10% is found in synaptic vesicles where it either exists in the free or bound state (Huidobro-Toro et al., 2008). In order to

elucidate the mechanism of action of loud noise and its relation to dopamine release and synthesis in the inferior colliculus, it is important that the changes that occur be assessed.

Neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Huntington's disease have been associated with iron accumulation (Leskovjan et al., 2009). Fe is an essential element involved in maintaining the delicate redox state in physiological systems (Leskovjan et al., 2009). It is mainly found bound by metalloenzymes in order to prevent oxidation (Leskovjan et al., 2009). Due to its use in electron transfer reactions, it is essential that its concentration is regulated (Leskovjan et al., 2009). Regulation is achieved by Fe mostly found bound to metalloproteins. It is therefore important to assess how noise impacts the concentration of Fe in the brain (Leskovjan et al., 2009).

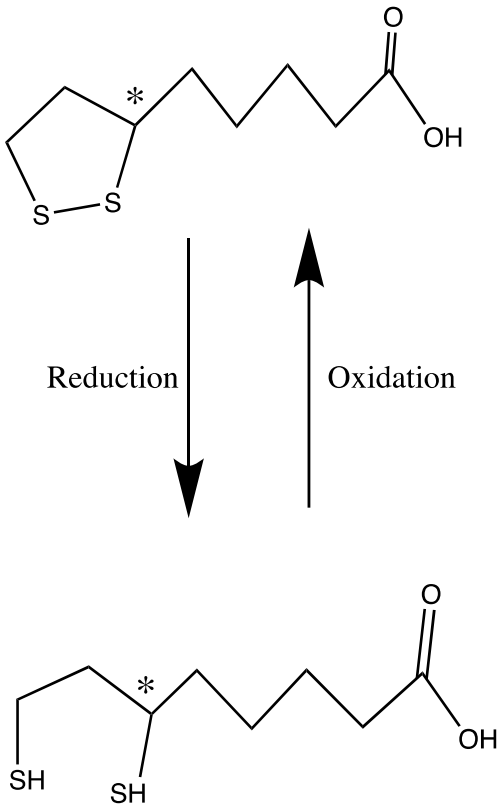
This current work will measure metal concentrations for Fe, Ca, K and Zn using X-ray fluorescent Spectrometry. Although the elements involved in neurotransmission are many, these elements were chosen due to their delicate roles in neurotransmission which may be disturbed upon noise exposure.

O4 Verifying if the Noise Induced Impact on the Dopamine System can be Prevented through Antioxidant, α-Lipoic Acid Administration.

Hearing loss can occur through the natural aging process, also known as presbycusis (Tavanai & Mohammadkhani, 2017), or through exposure to excessive noise (Park et al., 2014). An elevation in ROS has been implicated in both of these processes. Under normal conditions, ROS produced by the mitochondria are metabolized or scavenged by endogenous antioxidant systems (eg., catalase, Superoxide Dismutase and reduced Glutathione). However, either the aging process or excessive noise exposure can change this dynamic. This possibility has led to a search for antioxidants which can prevent or combat the generation of ROS in the auditory system. A recent study has shown that pre-treatment of mice with the antioxidant, methylene

blue before noise exposure significantly protected against cochlear injury (Park et al., 2014). Another study also indicates that α-lipoic acid may prevent ROS-induced mtDNA4834 bp deletion in the inner ear of rats (Peng et al., 2010).

α-lipoic acid



Dihydrolipoic acid

Reaction 1: The structure of α-lipoic acid. The molecule has a chiral carbon at position 6. Therefore, it can either exist in the R or S conformation. In the physiological environment it is reduced to Dihydrolipoic acid, which is the bioavailable form.

In the present study, we used α-lipoic acid as the antioxidant of choice. Alpha-lipoic acid was chosen because its bioavailable form, dihydrolipoic acid (whose formation is shown in Reaction 1), has a longer half-life *in vivo* and is well-tolerated by many mammals including rats (Peng et al., 2010). The administration of α-lipoic acid (intraperitoneal – i.p.) before noise exposure

was expected to offset the effects of ROS production in the inferior colliculus in response to the noise exposure. As a result, we expected the noise-induced effect to be alleviated by the α-lipoic acid administration. H_2O_2 was measured using the Horse Radish Peroxidase (HRP) colorimetric assay.

This experiment highlighted the effects of antioxidants in preventing ROS production. We expected that α-lipoic acid administration will ultimately prevent noise-induced changes in the neurotransmission of dopamine. The effect of α-lipoic acid administration on biometals was also examined by X-ray Fluorescent Spectrometry (XRF) to determine whether any noise-induced changes in concentration can be prevented.

In summary, the present work has expanded our understanding of the neural mechanisms underlying dopamine's role in the central auditory pathway and the dysregulation caused by exposure to loud noise.

CHAPTER II

LITERATURE REVIEW

Reactive Oxygen Species (ROS): Characteristics, Generation and Regulation

A reactive oxygen species can be defined as an oxygen-containing substance with one or more unpaired electrons in its valence orbitals that is capable of independent existence (Phaniendra et al., 2015). They are classified into two types: free radical and non-radical derivatives of oxygen. The free radical derivatives are superoxide ($^{\bullet}O_2^-$) and the hydroxyl radical ($^{\bullet}OH$), whereas the non-radical derivative is H_2O_2. The odd number of electron(s) of free radical ROS makes it unstable, short lived and highly reactive. The peroxide ion, O_2^{2-}, does not contain unpaired electrons however its oxidation state enables it to form stable bonds with two molecules of hydrogen, producing H_2O_2. Although H_2O_2 is a fairly stable molecule, under certain conditions such as the presence of Fe^{2+} ions it is easily converted into the $^{\bullet}OH$. Because of the high reactivity of ROS, they can abstract electrons from other compounds to attain stability. Thus, the attacked molecule loses its electron and becomes a free radical, beginning a reaction cascade that can damage a living cell (Phaniendra et al., 2015). In a physiological system, ROS exists predominantly as the non-radical H_2O_2 since it is more stable than its precursor, $^{\bullet}O_2^-$ and its conditional derivative, the $^{\bullet}OH$. A summary of the types of ROS and the process of formation is shown in the Figure 3 below.

Reactive oxygen species are normal by-products of oxidative phosphorylation in all cells, including brain cells. A significant amount of oxygen (O_2) consumed during the process is

converted into $^{\bullet}O_2^-$ which is the stoichiometric precursor of H_2O_2 (Chen et al., 2001). The reduction process of O_2 leads to the generation of the three types of ROS: H_2O_2, superoxide ($^{\bullet}O_2^-$) and the hydroxyl radical ($^{\bullet}OH$), as detailed in the schematic below. The enzyme, NOX reduces O_2 by a 1 electron (e^-) transfer to $^{\bullet}O_2^-$. $^{\bullet}O_2^-$ can also be produced as a result of cellular respiration in the mitochondrion. It is then rapidly dismutated to H_2O_2 and O_2 either spontaneously or by superoxide dismutase (SOD). Due to the fact that it is a free radical, $^{\bullet}O_2^-$ is highly reactive and the cell aims to keep its steady-state concentration low by rapidly converting it to H_2O_2. Hydrogen peroxide is considered a non-radical ROS and therefore is relatively more stable (Gulaboski et al., 2019). However, the O-O bond in H_2O_2 is sensitive to UV light, high temperatures, ionization radiation and metals such as Fe^{2+} (Fenton reaction, shown in reaction 2 below) and Cu^{2+}. Exposure to these conditions causes H_2O_2 to be converted to $^{\bullet}OH$ which is highly reactive and is responsible for oxidative damage to cells.

$$H_2O_2 + Fe^{2+} \longrightarrow {}^{\bullet}OH + Fe^{3+}$$

Reaction 2: Fenton reaction. In the presence of ferrous ion, H_2O_2 is converted to the hydroxyl radical and ferric ion.

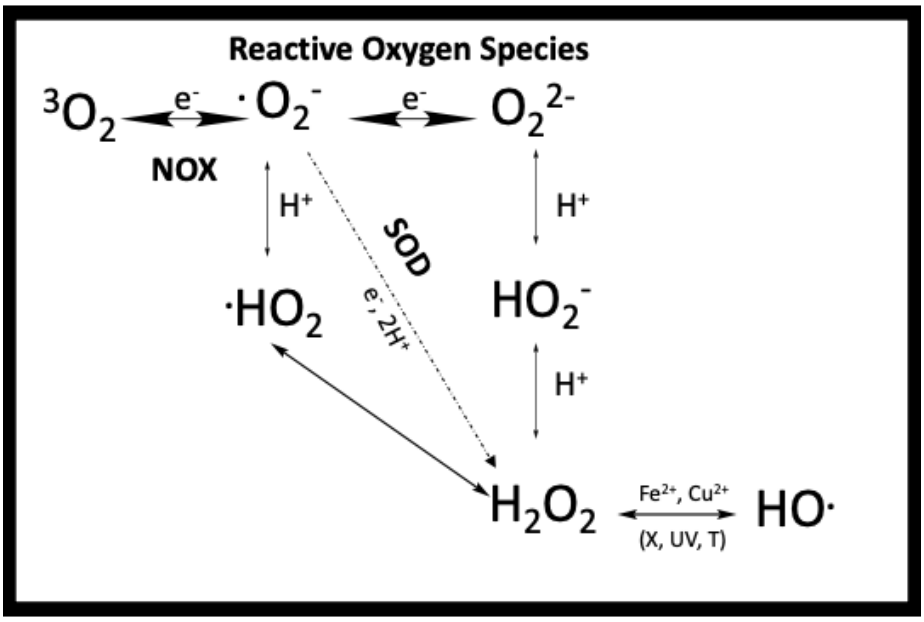


Figure 3: Formation of Reactive Oxygen Species. A general schematic showing the stepwise reduction (electron gain) of molecular oxygen into ROS. NOX – NADPH Oxidase. SOD – Superoxide dismutase.

Reactive oxygen species are a normal by-product of oxidative phosphorylation in the mitochondria of all living cells (Rice, 2011). Its concentration is regulated by the enzymes superoxide dismutase, catalase and glutathione peroxidase to prevent excessive build-up which is implicated in many pathological conditions (Chen et al., 2001). The schematic below (Figure 4) demonstrates the role these three enzymes play in detoxication of H_2O_2. Superoxide dismutase is an enzyme located in the mitochondria and acts as the first line of defense where it converts $^{\bullet}O_2^-$ to H_2O_2. The second line of defense involves catalase, located in the peroxisomes and glutathione peroxidase located both in the cytoplasm and mitochondria. They both perform the task of disproportionating H_2O_2 to water and oxygen. The buildup of either $^{\bullet}O_2^-$and/or H_2O_2 presents the potential of lipid peroxidation which left unchecked leads to oxidative damage of membranes as it is a self-perpetuating reaction (Jakoby, 2012). In fact, this explanation is believed to be responsible for the degradation of the myelin sheath observed in the neurons of

patients suffering from Parkinson's (Dean et al., 2016) and Alzheimer's diseases (Han et al., 2019). On the other hand, glutathione peroxidase mediates the prevention of further propagation of lipid peroxidation should it occur.

Reactive Oxygen Species (ROS) and its Pathological Effects in the Human Body

Reactive oxygen species exists in three main forms: O_2^-, $^\bullet OH$, and H_2O_2. Research has shown that O_2^- has a half-life of 10^{-4} to 10^{-6} seconds, $^\bullet OH$ has a half-life of 10^{-9} seconds and H_2O_2 has a half-life ranging from seconds to minutes (Casteilla et al., 2001). Therefore, at any point in time in the physiological environment, H_2O_2 will be the most predominant ROS. Due to the polarity of H_2O_2, it possesses the ability to cross the semi-permeable membranes of cells. Once in the cell, it unleashes its great oxidation potential thus destroying membranes, proteins and DNA (Jakoby, 2012) as shown in Figure 4. In the body, H_2O_2 undergoes a disproportionation reaction to produce H_2O and O_2, a process which is undertaken by the antioxidant regulatory network consisting of superoxide dismutase, catalase and glutathione peroxidase (Casteilla et al., 2001). Although this system exists, higher concentrations of H_2O_2 will take a longer time to undergo disproportionation. Therefore, its regulation is not only dependent on the antioxidant regulatory network but also on the concentration in a given cell. The buildup of H_2O_2 will enable its effects to be experienced at a location distant to where it was initially produced. At normal physiological concentrations, research has shown H_2O_2 to be a signaling molecule (Chen et al., 2001). However, at higher concentrations, H_2O_2 is toxic to living cells as it possesses the ability to oxidize biological macromolecules, thus, irreversibly altering the structure and function of DNA, protein and lipid structure (Figure 4) (Sanford et al., 2010). These alterations have been shown to lead to many disorders including neurodegenerative diseases such as Parkinson's and Alzheimer's diseases (Chen et al., 2001).

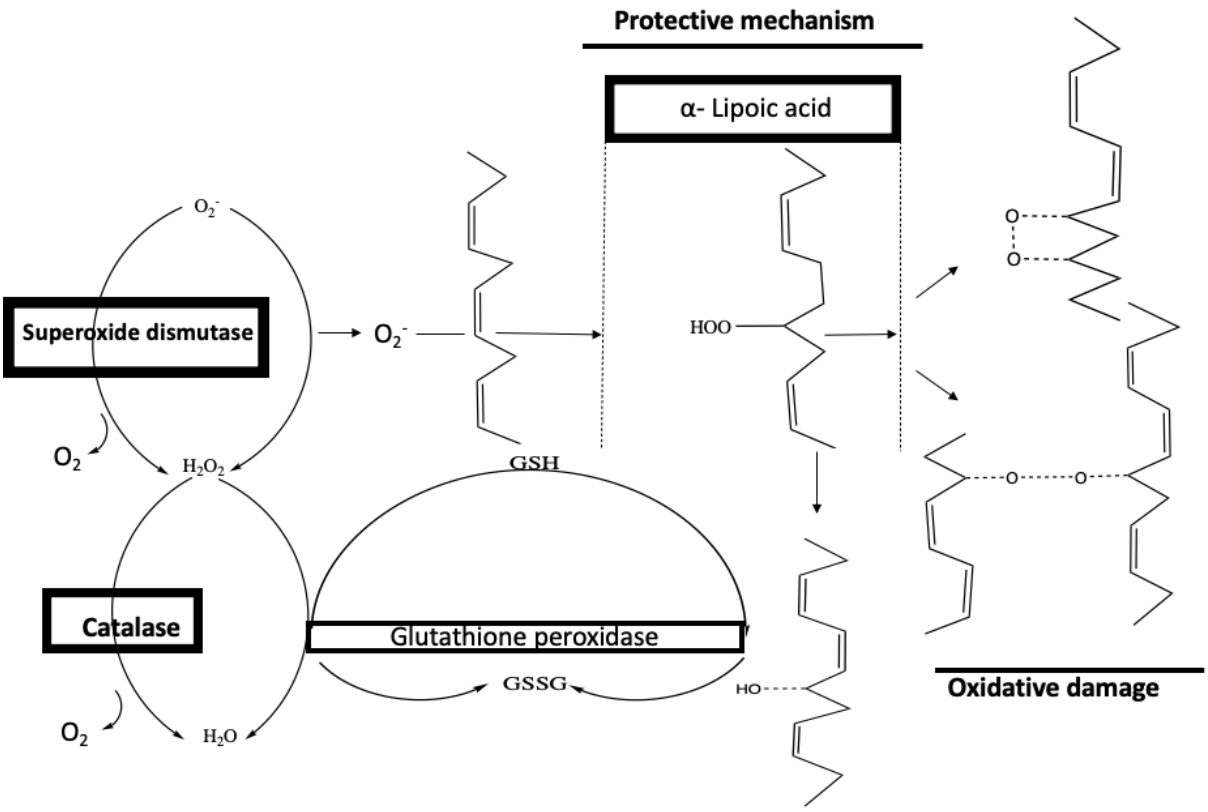


Figure 4: General illustration of the pathological effects of ROS and the three enzymes involved in the breaking down of hydrogen peroxide(Modified diagram from Jakoby, 2012)). The schematic shows the mechanisms by which ROS elicits oxidative damage which leads to its pathological effect. The conversion of the superoxide anion (O_2^-) into H_2O_2 is undertaken by Superoxide dismutase. The breakdown of H_2O_2 to water is undertaken by the enzyme's catalase and glutathione peroxidase. Glutathione peroxidase also prevents lipid peroxidation. α-lipoic acid is an antioxidant whose presence prevents the radicalization of membrane lipids.

Reactive Oxygen Species (ROS) and Hearing Loss

The auditory system works through a series of complex mechanisms ultimately resulting in the sense of hearing. It is divided into two systems: the peripheral and the central auditory systems. The peripheral system is responsible for the perception and relay of sound. It consists of the outer ear (pinna), middle ear and inner ear (cochlea).

Sound perception begins with the collection of sound waves by the pinna. Transmission of these waves occurs through the auditory canal until it reaches the eardrum. Vibration of the tympanic membrane causes a movement of the middle ear bones which consist of the malleus, incus and stapes. The sound waves are then processed into neural signals in the cochlea, which includes two kinds of hair cells: outer and inner hair cells. Hair cells are sensory receptors in the auditory system that possess the ability to detect movement in their environment through mechanotransduction. The outer hair cells are responsible for mechanical amplification of low-level sound that enters the ear (Schnupp et al., 2011). On the other hand, the inner hair cells are responsible for transforming sound vibrations in the cochlea into electrical signals which are then relayed to the auditory brainstem and to the auditory cortex (Schnupp et al., 2011). This event marks the end of processing in the peripheral system and marks the beginning of processing in the central auditory system.

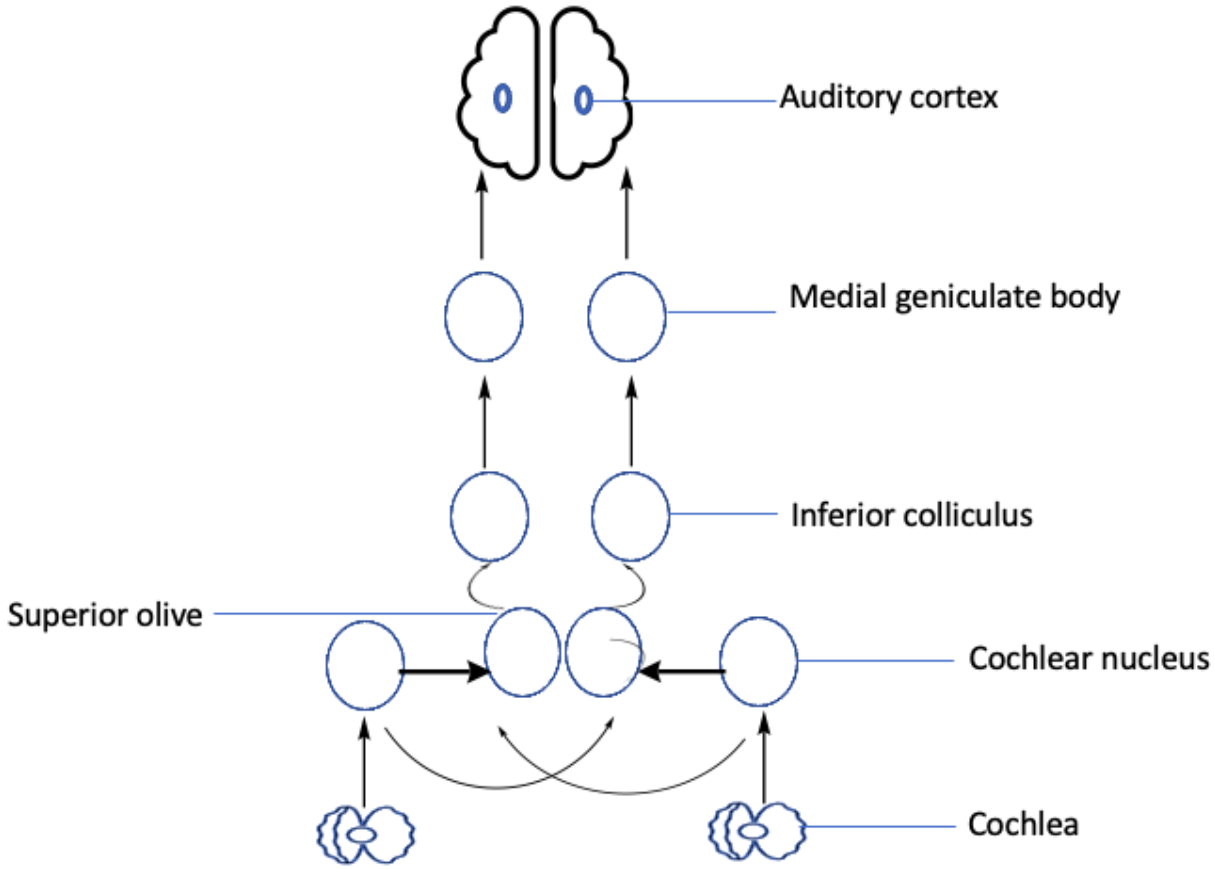


Figure 5: Schematic diagram showing the path of sound transmission from the cochlea which ultimately integrates at the inferior colliculus.

In the central auditory system (Figure 5), the neural signal is first received in the cochlear nucleus, which is responsible for signal decoding by discerning the frequency, duration and intensity of the sound wave (Peterson et al., 2020). Next, the signal is received by the superior olivary complex responsible for sound localization where majority of the auditory nerves cross the brainstem's midline. Finally, the connections between the contralateral cochlear nuclei with the ipsilateral superior olivary complex converge to form a single tract at the lateral lemniscus (Peterson et al., 2020). These fibers extend and converge in the inferior colliculus. At the inferior

colliculus, both ascending and descending pathways are conserved. The integration of auditory and non-auditory neural inputs converge here; thus, the inferior colliculus is deemed to be the call center of the central auditory system. From here, the neural information ends up at the relay center of the brain, called the thalamus, specifically the medial geniculate body, which is responsible for the integration of the sound in preparation for other actions. By the time processing reaches the auditory cortex, the sound has been decoded, recognized and memorized in preparation for a response (Peterson et al., 2020). This process of relay and interpretation of sound is achieved by the central auditory system as shown in Figure 5.

Reactive oxygen species produced as a result of excessive noise exposure induces hair cell death and damage to cochlear tissue (Henderson et al., 2006). In fact, a significant body of evidence has shown that ROS elicit extensive damage to the peripheral auditory system. A research study showed that a long-term high fat diet increases oxidative stress, mitochondrial damage and apoptosis in the inner ear of D-galactose aged rats. Thus, excessive ROS produced as a result of high fat diet increased caspase-3 mediated apoptosis in the inner ear which is a part of the peripheral auditory system (Du et al., 2012).

Currently, research that focuses on the impact of ROS in the central auditory system trails that of the peripheral auditory system. However, because hearing involves a complex interplay of both peripheral and central auditory signalling, the elucidation of the mechanisms resulting in hearing loss should not only focus on the peripheral auditory system but also on the central auditory system. Although, some progress has been made, research is currently lacking on the impact of ROS on the central auditory system. For example, it has been recently shown that NADPH dependent oxidative stress can be induced by prematurely aging rats using D-galactose (Du et al., 2012). Using this model of premature aging, the study found that levels of H_2O_2 and

the expression of NADPH oxidase 2 (NOX2), two biomarkers of oxidative stress, were greatly increased in the ventral cochlear nucleus thus activating the mitochondrial apoptotic pathway in the ventral cochlear nucleus (Du et al., 2015). Furthermore, another recent study examined the apoptotic damage to the dorsal cochlear nucleus, the ventral cochlear nucleus and the inferior colliculus (Fröhlich et al., 2018). Although it was determined that excessive and prolonged noise exposure had a long-lasting effect of apoptosis in these compartments of the central auditory pathway (Fröhlich et al., 2018), there was no mention of the underlying mechanism by which apoptosis is initiated. In order for apoptotic pathways to be activated, there must be an underlying mechanism that is responsible. Inasmuch as apoptosis seems to be a heavily explored area when it comes to hearing loss, research is lacking concerning the other pathways that may be involved and how these are linked. Therefore, the current research seeks to bridge the gap by exploring the signalling mechanism that may be mediated by ROS.

There is the need for extensive research on the impacts of ROS on the central auditory system, specifically the inferior colliculus which is the principal integration center for hearing and its related processes. The integration of auditory and non-auditory neural inputs converge here, thus the inferior colliculus serves as a major center of integration in the ascending as well as descending auditory pathways (Peterson et al., 2020). Both excitatory and inhibitory amino acid neurotransmitters including dopamine have been linked to the auditory processes in the inferior colliculus, though the specific role of dopamine here remains elusive (Hoyt et al., 2019).

Hydrogen Peroxide (H_2O_2) and Synaptic Dopamine Transmission in the Inferior Colliculus

Dopamine is a neurotransmitter whose role in learning, memory, cognition, voluntary movement, and reward-related behaviors has been well-studied. However, it is not typically associated with hearing or auditory processes. In spite of this, current research has uncovered a significant amount of convincing data that suggest dopamine's role in auditory processes. For example, changes in TyrH and dopamine receptor gene expressions have been reported in the dorsal cortex and the central nucleus of the inferior colliculus following bilateral deafening (Tong et al., 2005). Furthermore, electrophysiological experiments recorded in the inferior colliculus of live mice have shown that dopamine altered neuronal response in a heterogeneous manner (Gittelman et al., 2013). Thus, dopamine affects auditory responses in a context-specific manner, meaning that the role it plays depends on the system in which it is acting (Gittelman et al., 2013). Concomitant with the research by Tong et al, a recent study also indicates that changes in TyrH and dopamine receptor gene expressions persist in the cochlear nucleus and inferior colliculus, following acoustic trauma (Fyk-Kolodziej et al., 2015a). Although this evidence exists, the mode and function of dopamine neurotransmission in the auditory system, particularly in the inferior colliculus remains unclear.

While the exact role of dopamine in hearing and its related noised-induced disorders is not apparent, a significant amount of evidence exist that implicates ROS in the dysfunction of the dopamine system that may underlie such disorders. Hydrogen peroxide is a signalling molecule which modulates the synaptic release of many neurotransmitters (Rice, 2011). Previous studies have shown that endogenous H_2O_2 modulates dopamine release through glutamate and gamma-aminobutyric acid (GABA) neurotransmitters (Avshalumov et al., 2003). Furthermore, H_2O_2 has

been shown to downregulate the synaptic dopamine release through calcium channels (Chen et al., 2001). Two mechanisms have been proposed to be responsible for this observation.

Firstly, it has been suggested that excessive H_2O_2 production causes depletion in adenosine triphosphate (Rice, 2011). Consequently, enzymes such as phosphorylases and kinases that are dependent on ATP will be deprived of their cofactors. These phosphorylases and kinases interact by G-protein coupled receptors to control the opening and closing of calcium channels (Ford, 2014). Thus, the inactivation of these phosphorylases and kinases will result in the inability to interact with G-protein coupled receptors because conformation is key when it comes to protein-receptor interactions. Due to the lack of protein-receptor interaction, the calcium channels that modulate dopamine release will be shut off (Ford, 2014). Secondly, it has been proposed that the presence of H_2O_2 elicits oxidative modifications of enzymes such as tyrosine hydroxylase required for the synthesis of dopamine (Daubner et al., 2011). The oxidative modifications include nitration and thiolation of amino acids in or near the enzyme active site (Daubner et al., 2011). Nitration occurs at three tyrosine residues located in a flexible loop close to the enzyme active site (Daubner et al., 2011), whereas thiolation occurs at six of the seven cysteine residues that TyrH possesses (Daubner et al., 2011). Enzymes in general are active when the right conformation can be attained, and this is no different for TyrH. Nitration and thiolation of these essential amino acids in its enzymatic structure causes TyrH to lose its native conformation and it is therefore deactivated. Consequently, there will be a shutdown in dopamine production since TyrH is the rate-limiting factor in the synthesis of dopamine. Connecting these two mechanisms is the fact that cysteine residues on TyrH have been shown to be selective targets of H_2O_2-induced oxidation. Thus, it has been proposed that H_2O_2 and other cellular oxidants are physiological redox regulators of TyrH (Denu & Tanner, 1998).

Hydrogen Peroxide (H_2O_2) and its Relationship with Adenosine Triphosphate (ATP) Generation

Although ROS is a normal by-product of cellular respiration, at high concentrations H_2O_2 possesses the ability to deplete the amount of ATP produced from the oxidative phosphorylation mechanism. The mechanism by which this happens is explained later in this section. It is for this reason that the cell employs a number of mechanisms to facilitate the breakdown of H_2O_2. These mechanisms include spontaneous degeneration and enzymatic breakdown. At high concentrations, there is diffusion from one cellular compartment to the mitochondrion of another cell or the same cell to facilitate rapid enzymatic breakdown to water. The enzyme involved in the mitochondrial breakdown of H_2O_2 to water is glutathione peroxidase (Casteilla et al., 2001). The reaction mechanism first requires the conversion of reduced glutathione in the presence of H_2O_2 to oxidized glutathione. This reaction must then be coupled to the oxidation of NADPH in order to regenerate the reduced glutathione which is a co-substrate in the reduction of H_2O_2. The details of the reaction are shown below (Reaction 3).

$$H_2O_2 + 2\ GSH\ (\text{Reduced glutathione}) \xrightarrow[(\text{mitochondria, cytosol})]{\textit{Glutathione Peroxidase}} 2H_2O + GSSG\ (\text{Oxidized glutathione})$$

$$GSSG + NADPH + H^+ \xrightarrow[(\text{mitochondria, cytosol})]{\textit{Glutathione reductase}} 2GSH + NADP^+$$

Reaction 3: Glutathione peroxidase requires reduced glutathione as a co-substrate in the breakdown of H_2O_2 to H_2O. Reduced glutathione must then be regenerated from oxidized glutathione, a reaction catalyzed by glutathione reductase which also requires NADPH as a co-factor.

NADPH, which is an important precursor in many biosynthetic pathways, is diverted to facilitate the breakdown of H_2O_2. Moreover, NADPH when oxidized in the oxidative phosphorylation mechanism produces an equivalent of approximately 3 ATP molecules. Thus, this diversion also depletes the cell of the ATP that is expected to be produced from NADPH. The normal physiological concentration of H_2O_2 is dependent on the cellular compartment and the system to which the cell belongs. The intracellular concentration of H_2O_2 in a given cell is dependent on the balance between its production and removal. The tolerable level represents the range that the cell can effectively regulate to prevent a decrease in ATP production in the process of oxidative phosphorylation. Since the normal physiological concentration remains controversial in literature and is also different for every system, an estimate of 0.1-100 μM will be used as physiological cut-off based as determined in the liver in previous literature (Sies, 2017). The actual levels of H_2O_2 in the inferior colliculus will be determined in this work. Concentrations greater than the normal physiological level will proportionally cause greater loss of ATP through the diversion of NADPH.

The Role of Metals in Neurotransmission

Inasmuch as proteins play a huge role in neurotransmission, certain elements such as $Ca^{2+,}$ K^+, Fe^{2+} and Zn^{2+} also control many vital processes (Huidobro-Toro et al., 2008). It has been demonstrated that Ca^{2+} and K^+ control the voltage gated channels that allow the release of dopamine (Chen et al., 2001). Furthermore, the activation of tyrosine hydroxylase proceeds via Ca^{2+} dependent phosphorylation mechanism (Daubner et al., 2011). Aside from these roles, Ca^{2+} has been shown to be involved in calcium activated signaling (Rice, 2011). An altered concentration of Ca^{2+} has been implicated in the protein aggregation characteristic of

Alzheimer's (Benedictis et al., 2019), Parkinson's and Huntington's diseases (Grochowski et al., 2019). It is clear that Ca^{2+} homeostasis is an essential process in neurotransmission. Defective neurotransmission may result when the concentrations of biometals are imbalanced. As the name suggests, trace metals such as Fe and Ca confer protective roles against oxidative damage when in small quantities (Grochowski et al., 2019). They also function in regulating enzyme catalysis, gene expression and function as second messengers in the presence of ROS. Therefore, the increase in physiological concentrations of trace metals may be indicative of oxidative stress/damage or pathological conditions (Grochowski et al., 2019). Although some progress has been made to discover the role of trace metals in neurodegenerative diseases, the mechanisms through which noise may impact the physiological concentration of trace metals such as Fe, Ca, K and Zn ions in the central auditory system remains elusive. Zinc is an important biometal which coordinates many neurological proteins, thus playing an important role in neurotransmission. As part of this project, analysis of Fe, Ca, K and Zn concentrations will be compared between three experimental groups: control rats, noise exposed rats and rats administered α-lipoic acid prior to noise exposure.

Measurement of Hydrogen Peroxide (H_2O_2)

Hydrogen peroxide has great oxidation potential due to the fact that it is an incompletely reduced metabolite of oxygen (Rhee et al., 2010). For this reason, it has various physiological and pathological effects in living cells. Characterization of the functions of H_2O_2 requires selective measurement in the presence of other oxygen metabolites with spatial and temporal fidelity (Rhee et al., 2010). Various colorimetric assays have been developed for the effective and relatively rapid measurement of intracellular H_2O_2. Some of these assays have been designed based on redox properties where H_2O_2 oxidizes the active ingredient in the assay reagent. For

example, H_2O_2 in the presence of horse-radish peroxidase (HRP) reacts stoichiometrically with Amplex red, the active ingredient, to generate a red fluorescent oxidation product called resorufin (Rhee et al., 2010). Amplex red has the advantage of being a highly sensitive and stable substrate for HRP with selectivity for H_2O_2. Therefore, its main disadvantage is the use of HRP which makes it a partially enzymatic assay therefore care must be taken about the temperature and pH of the reaction media.

Another method that has been used frequently in the analysis of hydrogen peroxide is an assay developed by National Diagnostics. This assay depends on the ferrous oxidation in the presence of xylenol orange and is commonly referred to as the FOX method. In the presence of H_2O_2, Fe^{2+} will be oxidized to Fe^{3+}. Fe^{3+} then reacts with the active ingredient, Xylenol orange to form a blue-purple complex, o-cresolsulfone-phthalein 3′,3″-bis(methylimino) diacetate iron (III), measurable at 560 nm with an extinction coefficient of $1.5 \times 10^4 M^{-1}cm^{-1}$ (Rhee et al., 2010). This assay has the advantage of being able to detect low concentrations of H_2O_2 in aqueous media, detecting levels as low as 15 ng/mL. Also, the FOX assay is relatively free from interference from proteins and salts. Its sole disadvantage is that chelating agents such as ethylenediamine tetraacetic acid that bind Fe^{3+} will interfere with the assay (Rhee et al., 2010).

The HRP colorimetric assay was used in the present study to examine whether or not H_2O_2, or ROS in general, plays a role in noise effects on the dopamine system in the inferior colliculus. This assay was chosen due to its high sensitivity and selectivity. To minimize possible enzymatic degradation of HRP, the enzyme will be kept on ice throughout the duration of the assay.

CHAPTER III

MATERIALS AND METHODS

The methods described in this section include HRP enzymatic assay, ATP colorimetric assay and metal ion analysis using X-ray fluorescence spectrometry (XRF).

Chemicals and Materials

Horse radish peroxidase enzymatic assay was purchased from ABCAM, USA. Adenosine triphosphate colorimetric assay and α-lipoic acid were purchased from Sigma-Aldrich, USA. 10 kDA MWCO filters were purchased from Sartorius, USA**.**

Animals

Both male and female adult Sprague-Dawley rats between the age of 8–12 weeks old were used in this study to maintain consistency with research performed in the field. All animal procedures were carried out in accordance with *the Guidelines for the Care and Use of Laboratory Animals* as approved by the University of Northern Colorado's Institutional Animal Care and Use Committee (IACUC). The rats were divided into two experimental groups. The first group was not exposed to noise and was considered the control group. The second group was the noise exposed group.

Noise Exposure Model

The noise exposure model that was used has been well characterized and has been shown to elicit permanent hearing loss. This noise model has been widely reported in literature (Luo et al., 2014; Muca et al., 2018; Yang et al., 2007). Freely moving adult Sprague-Dawley rats were exposed to noise in a sound-proof booth through three overhead speakers. The noise was administered by a computer-controlled signal processing software called Daqarta®, with the intensity of 118 dB sound at 1/3 octave band for four hours. The rats were removed from the booth following the exposure and sacrificed 24 hours later via carbon dioxide asphyxiation. The 24-hour wait time before sacrifice was allowed to ensure consistency with previous work from our lab and also to ensure that changes observed are not transitory but are long-term effects of the noise exposure. The brain tissues from these rats were quickly removed and processed for colorimetric measurement of H_2O_2 by the HRP assay, ATP colorimetric measurement and metal ion analysis using XRF.

Antioxidant (α-Lipoic Acid) Administration

In order to highlight the role of ROS in the proposed mechanism, α-lipoic acid was used to determine its effect on the noise induced alterations in the inferior colliculus. Rats were divided into two groups and administered 50 mg/kg α-lipoic acid intraperitoneally. One group was exposed to noise 24 hours after I.P injection of α-lipoic acid as in the protocol previously discussed. The second group was allowed a 1-hour recovery period and then exposed to noise in the manner previously discussed in the section above. 24 hours following the noise exposure, the rats were sacrificed, and their brains processed for colorimetric measurement of H_2O_2 as well as ATP measurements. The injection was controlled for by administering 1 ml of 0.9% saline to two groups of rats. Similar to the test group, the saline control group was split into two groups:

one group was allowed a 24-hour recovery before noise exposure while the other was allowed a 1-hour recovery before noise exposure. Hydrogen peroxide and adenosine triphosphate levels were compared among four experimental groups namely: i) control, ii) noise exposed without prior treatment iii) noise exposed after antioxidant administration and iv) noise exposed after saline administration.

Processing of the Brain Tissues from the Inferior Colliculus for Hydrogen Peroxide (H_2O_2) Assay

Following the noise exposure, the inferior colliculus was carefully dissected from the rat brains and placed in a 2-mL Eppendorf tube and then quick frozen in liquid nitrogen. The brain samples were subsequently stored in a -80 ^oC freezer till the day of protein extraction.

The inferior colliculus samples were washed with ice cold Phosphate-buffered saline (PBS) prior to processing. The tissue samples were resuspended in 1000 µl of H_2O_2 assay buffer on ice. The mixture was then homogenized until the tissue was completely smooth and fully suspended in the buffer. The suspension was then centrifuged at 12,000xg for 10 minutes in a refrigerated centrifuge kept stable at 4°C. After this, the supernatant was separated from the pellet using a micropipette. The supernatant was then deproteinized using a 10kDa molecular weight cut-off (MWCO) filter and then stored at -80 °C for use in subsequent experiments.

Colorimetric Assessment of Hydrogen Peroxide (H_2O_2) Levels in Brain Tissues

Intracellular changes in ROS were evaluated in noise exposed rats versus their control counterparts using the HRP colorimetric H_2O_2 assay kit obtained from ABCAM. The assay works on the principle that H_2O_2 in the presence of horse-radish peroxidase (HRP) reacts stoichiometrically with the active ingredient, Amplex red to generate a red fluorescent oxidation product, resorufin (Rhee et al., 2010) which can be detected at 570 nm. The measurement of

hydrogen peroxide was done using the external calibration method. The external calibration method involved standards of various concentrations spanning a range of concentrations 1 nM to 5 nM. The signal from each of these standards was measured using the SpectraMax 190 Microplate Reader and a calibration curve constructed. The signal from the brain samples was also measured and the concentration of H_2O_2 determined from the equation of the line.

Colorimetric Analysis of Adenosine Triphosphate (ATP)

Adenosine triphosphate levels were measured using the ATP Colorimetric/Fluorometric assay kit developed by Sigma-Aldrich®. 50μL standards/samples were prepared using the assay components described in the assay protocol. Standards spanned a concentration range of 0.02mM to 0.1 mM and were prepared from the ATP standard solution provided in the kit. The homogenized samples were deproteinized using a 10kDa molecular weight cut-off (MWCO) filter. Samples were diluted 1 in 10, 1 in 100 and 1 in 1000 dilution factors to ensure that the readings were within the range of the standard curve. The absorbance produced was measured in the SpectraMax 190 Microplate Reader at 587nm. Concentrations were read from an external calibration curve as described in section 3.1.4 above.

X-Ray Fluorescence Analysis of Metal Ions in Brain Tissue Samples

Tissue samples from the inferior colliculus were weighed for 5 samples/group. The samples were divided into three experimental groups, namely Control, Noise exposed and Lipoic acid+ Noise exposed groups. Sample lysates were prepared by homogenizing pre-weighed tissue in 700 μL 50% w/v HNO_3 and 300 μL 3% H_2O_2. Samples were spiked with Ga standard to a concentration of 1 mg/L Ga. 10 μL of sample was pipetted onto the middle of a sample disc and oven-dried, loaded into the XRF instrument and the elemental analysis performed.

Data Analyses

All data were analyzed using Graphpad Prism Software. Statistical analysis was performed for H_2O_2 and ATP data sets by non-parametric t-test (2 comparisons per data set) and one way ANOVA (3 or more comparisons per data set). Statistical analysis was performed for biometal analysis using One tailed sample t and Wilcoxon test (2 comparisons per data set). The criteria for statistical significance for all concentrations was set to $P < 0.05$. All data was reported as mean ± standard error of the mean (SEM).

CHAPTER IV

RESULTS

Assessing the Impact of Loud Noise on Production of Reactive Oxygen Species (ROS), Specifically Hydrogen Peroxide (H_2O_2) in the Inferior Colliculus

To assess the impact of loud noise on the production of hydrogen peroxide in the inferior colliculus, the HRP assay was performed as seen in Figure 6A. The calibration curve constructed showed a positive linear relationship between concentration and absorbance with an R^2 value of 0.9982 (Figure 6B). Upon performing the assay, it was revealed that the noise exposed groups showed a significant increase in ROS as compared to control. The mean for the control (n =10) was 0.15 nM while that for the noise exposed group (n =10) was 0.37 nM, indicating a more than two-fold increase with a $p < 0.0001$ in H_2O_2 concentration as shown in Figure 6C.

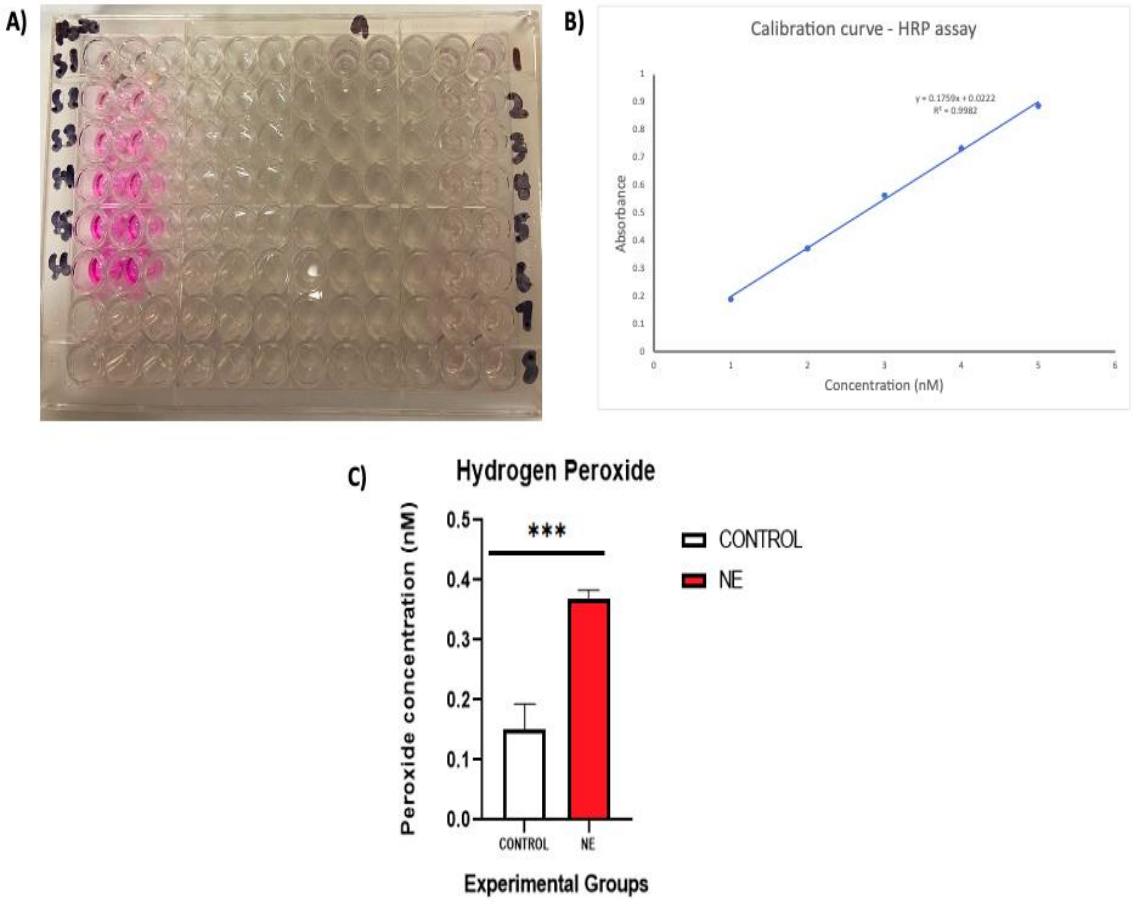


Figure 6: Concentration of H_2O_2 before and after noise exposure. A) 99 well plate containing H_2O_2 standards in the first two rows. B) Calibration curve for Horse Radish Peroxidase assay, R^2 value = 0.9982 C) Changes in H_2O_2 levels upon noise exposure. Statistical significance determined by non-parametric t-test, $p < 0.0001$.

Examining the Effect of Noise on Adenosine Triphosphate (ATP) Levels in the Inferior Colliculus

To explore how noise induced changes in H_2O_2 could modulate dopamine neurotransmission as seen in Figure 7A, ATP levels in the inferior colliculus were examined following noise exposure and compared to the control. A linear relationship between concentration of ATP standards and absorbance was obtained with an R^2 value of 0.9984 as seen in Figure 7B. Upon performing the assay, it was revealed that there was no significant difference between the noise exposed group and the control ($p = 0.5805$, $n = 10$/group). The mean for the control group was 1.74 nM while the mean for the noise exposed group was 1.38 nM, as seen in Figure 7C.

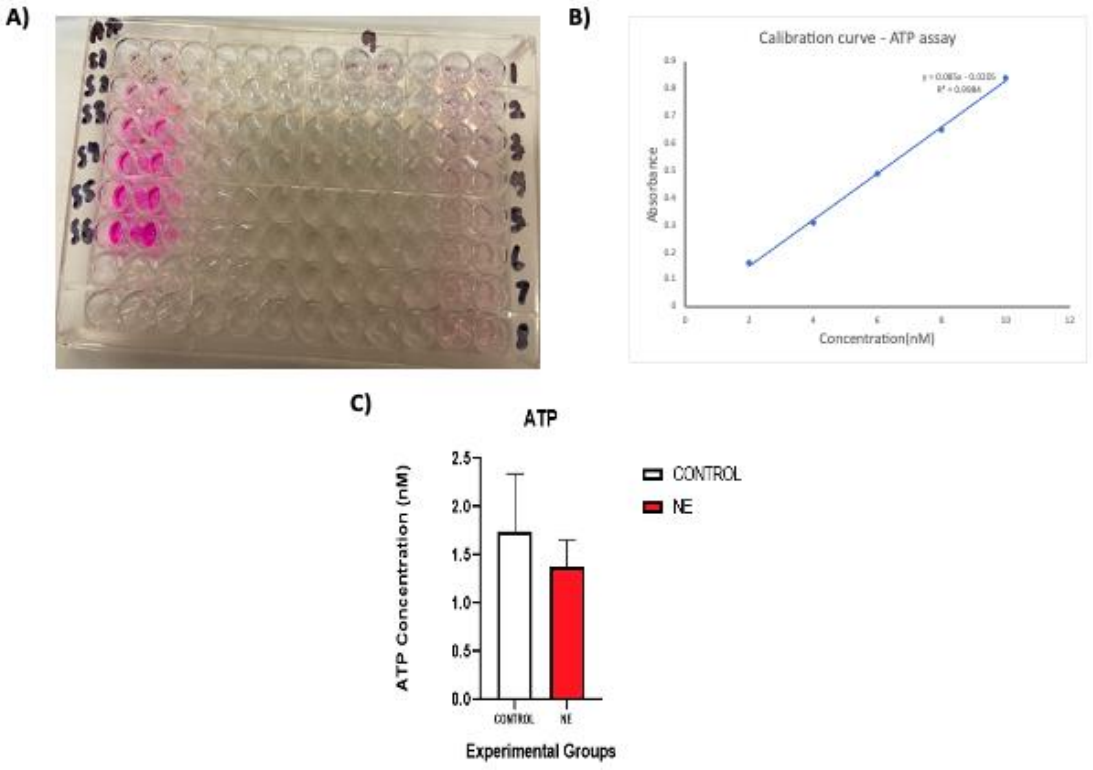


Figure 7: Examining the effect of noise on ATP concentrations in the inferior colliculus. A) 99 Well plate containing ATP standards in the first two rows. B) Linear calibration curve for ATP measurement, R^2 value = 0.9984. C) ATP levels compared between noise exposed and control groups. Statistical significance determined by non-parametric t-test, p value = 0.5805. NE: Noise exposed.

Assessing the Impact of Noise Exposure on the Biometals Involved in Neurotransmission

To assess the impact of noise exposure on biometals involved in neurotransmission, XRF analysis was performed for Fe, Ca, K and Zn ions. X-ray fluorescent spectra and the concentrations of these metal ion were obtained for each run. Comparing the spectra for the two experimental groups revealed elevated intensities for K and Ca ion peaks in the noise exposed subjects compared to their controls (Figure 8). The intensity of the Fe and Zn peaks remained relatively the same between the control and noise-exposed groups. Other ions detected in the sample were Cu, S, P and Mo. The observed peak intensities for the ions were proportional to their respective concentrations.

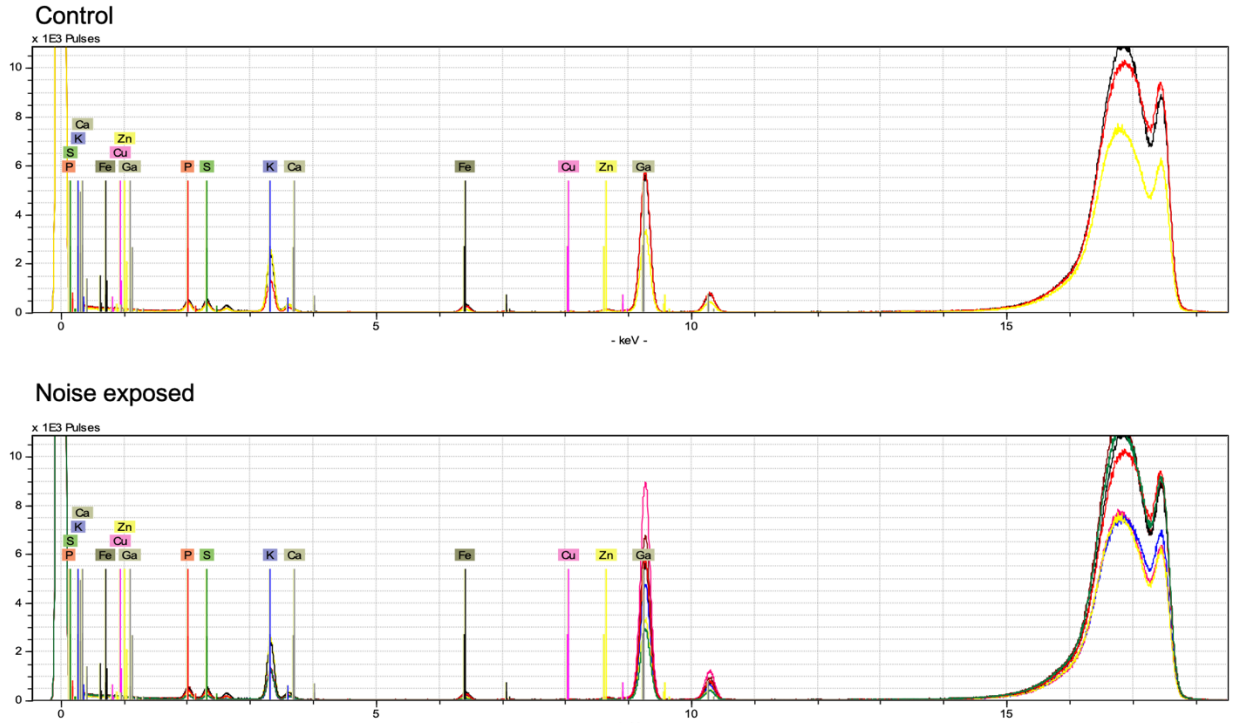


Figure 8: Representative XRF spectra for control and noise exposed groups. Each peak corresponds to an element detected in the sample. The intensity of the peak corresponds to the concentration of the specific element in the sample.

The concentrations of the ions were normalized to the weight of the tissue sample and expressed as μg/g. A Q test was performed to exclude any outliers in the data set. Statistical analysis (One sample t and Wilcoxon test) was also performed to determine significance in the ion concentrations between the experimental groups. The results obtained showed a significant increase in Fe concentration ($p = 0.0414$) in the noise exposed subjects (mean = 0.0003 μg/g) compared to their controls (5.1×10^{-5} μg/g; Figure 9A). Ca also increased significantly from a mean of 0.0002 μg/g in the control subjects to 0.0013 μg/g in comparison to in their noise exposed counterparts (Figure 9B), with a p value of 0.0115. Furthermore, K increased significantly from a mean of 0.0004 μg/g in the control subjects to 0.003 μg/g in their noise exposed subjects with a p value of 0.0380 (Figure 9C). However, there was no significant difference in Zn concentration between the two experimental groups ($p = 0.0645$), where the control subjects had a mean Zn concentration of 3.8×10^{-5} μg/g and that the noise exposed group was 5.3×10^{-5} μg/g (Figure 9D).

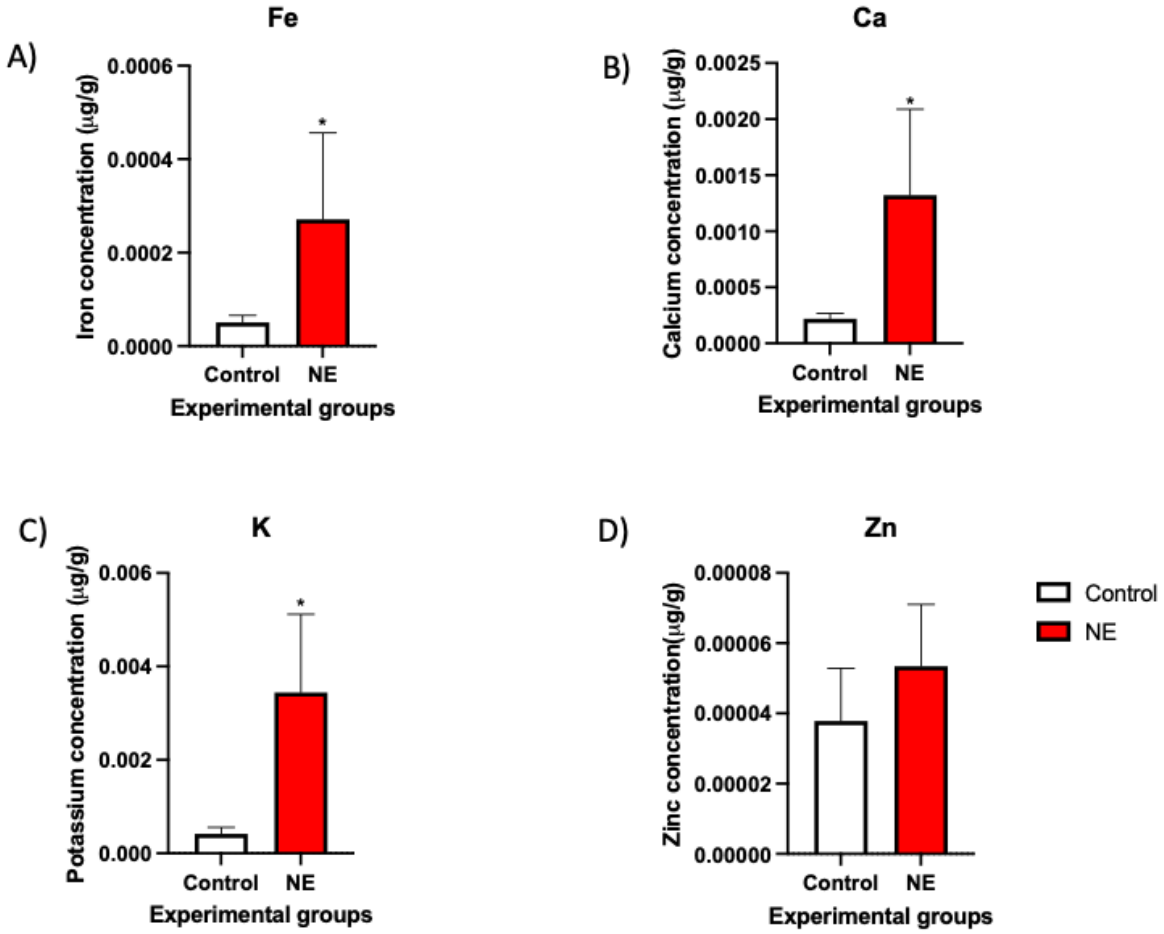


Figure 9: Effects of noise exposure on the concentrations of biometals: Fe, Ca, K and Zn. A) Increase Fe concentration compared to their control counterpart. B) Increase in Ca levels observed in noise exposed subject relative to that of the control. C) Increase in K concentrations in noise exposed group versus their controls. D) No significant change in Zn levels in the two experimental groups. Statistical significance determined by one sample t and Wilcoxon test. *P < 0.05. NE: Noise exposed.

Verifying if the Noise Induced in the Inferior Colliculus can be Prevented through Antioxidant, α-Lipoic Acid Administration

To verify if the noise-induced changes in the inferior colliculus can be prevented through the administration of α-lipoic acid, a comprehensive analysis of H_2O_2, ATP and biometal levels was undertaken in noise exposed subjects versus their controls.

A one way ANOVA analysis with Tukey's post hoc test was done for all data sets in the experimental group. Comparison between the noise-exposed (n = 10) and lipoic acid + noise exposed (n = 9) groups showed a decrease in the concentration of H_2O_2 concentration from 0.37 nM in the noise exposed group to 0.18 nM in the lipoic acid + same day noise exposed group, with a p value < 0.0001 as shown in Figure 10A. Further comparison of the lipoic acid + noise exposed group to the control group showed no significant differences in H_2O_2 concentration between the two groups (p-value = 0.9793). Comparison between the noise-exposed versus lipoic acid + noise exposed 24 hours later group showed a borderline significance in H_2O_2 levels between the two groups, with the noise-exposed group has a mean of 0.37 nM while the lipoic acid + noise exposed 24 hours later group having a mean of 0.23 nM as shown in Figure 10B (p = 0.0643). When testing for the efficacy of α-lipoic acid as an effective antioxidant, the α-lipoic acid injection was controlled for with saline injection. The lipoic acid group showed a significant reduction in H_2O_2, from a mean of 0.42 nM in the saline group to a mean of 0.18 nM in the α-lipoic acid group with a p value < 0.0001 as shown in Figure 10B. The effectiveness of α-lipoic acid was tested 24 hours later by comparing the α-lipoic acid + noise exposed 24 hours later group to the saline + noise exposed 24 hours later group. There were no significant differences in H_2O_2 levels between the two groups (p = 0.9411). However, comparison of the lipoic acid + noise exposed 24 hours later group (mean = 0.23 nM) to the saline + noise exposed group (mean

= 0.42 nM) showed a significant increase in H_2O_2 concentration in the saline + noise exposed group with a p value of 0.0037.

Adenosine Triphosphate concentration comparisons between the noise-exposed (mean =1.38 nM) versus lipoic acid + noise exposed on the same day group (mean = 0.53 nM) revealed no significant differences between the two groups as shown in Figure 10C ($p = 0.1622$). Further comparison of the control group versus the lipoic acid + noise exposed 24 hours later also showed no significant difference between the two groups ($p = 0.9882$) with the control group having a mean of 1.74 nM while the lipoic acid + noise exposed 24 hours later had a mean of 1.39 nM as shown in Figure 10C. Mean- to-mean comparisons between the noise-exposed versus the lipoic acid + noise exposed 24 hours later groups showed no significant differences in ATP levels between the two groups ($p > 0.9999$), with the noise-exposed having a mean of 1.38 nM and the lipoic acid + noise exposed 24 hours later group, 1.39 nM as shown in Figure 10D. While testing the efficiency of α-lipoic acid to restore ATP levels, α-lipoic acid injection was controlled for with saline injection before the noise exposure. The results showed no significant differences in ATP levels between the two groups ($p > 0.9999$) with the α-lipoic + noise exposure on the same day acid having a mean of 0.53 nM while the saline-injected + noise exposure on the same day had a mean of 0.56 nM as shown in Figure 10D.

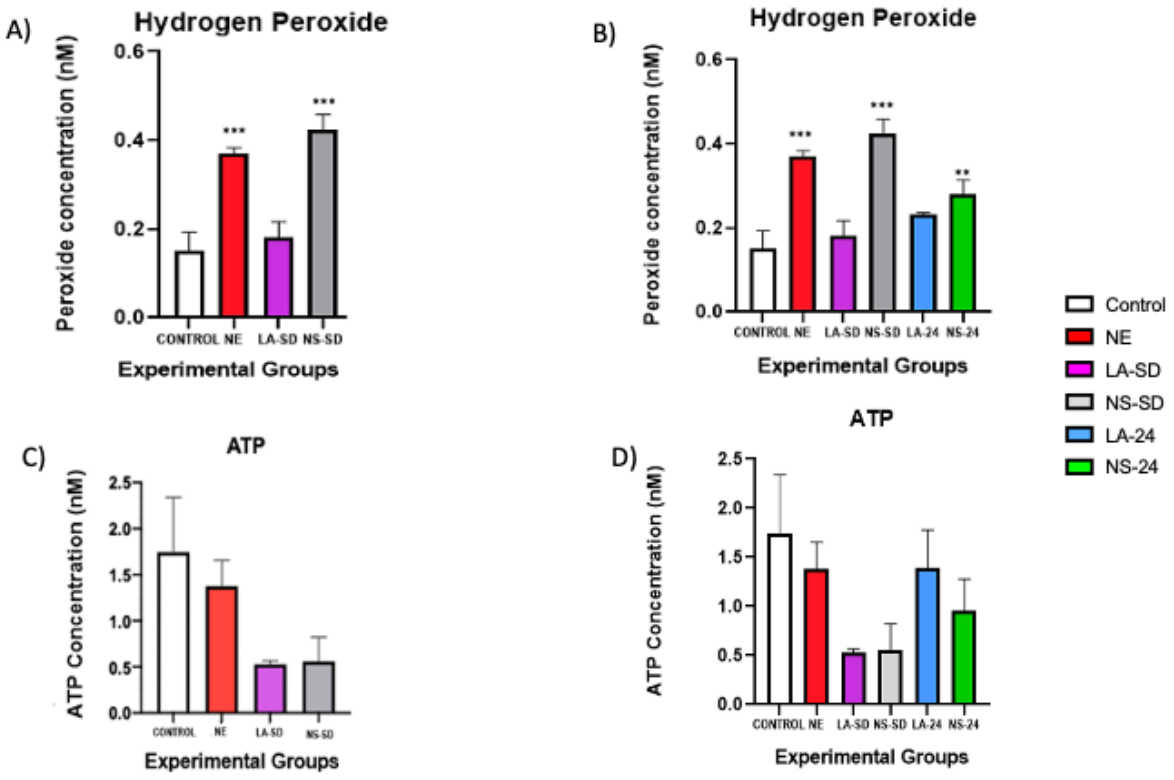


Figure 10: Effects of α-lipoic acid administration on H_2O_2 and ATP levels. A) Decrease in H_2O_2 in the group administered α- lipoic acid before noise exposure, $p < 0.0001$. B) Downward trend in H_2O_2 levels observed in group administered α- lipoic acid 24 hours before noise exposure. C) No change in ATP levels in group administered α- lipoic acid immediately before noise exposure ($p = 0.1622$). D) No change in ATP levels in group administered α- lipoic acid 24 hours before noise exposure ($p = 0.9822$). NE: Noise exposed. LA -SD: α- Lipoic acid administered immediately before noise exposure. NS – SD: Normal saline administered immediately before noise exposure. LA-24: α- Lipoic acid administered 24 hours prior to noise exposure. NS – 24: Normal saline administered 24 hours prior to noise exposure.

To assess how α-lipoic acid administered prior to noise exposure mitigates the noise-induced effect on the biometals involved in neurotransmission, XRF analysis was performed for Fe, Ca, K and Zn ions. Comparison of representative spectra between the control and α-lipoic + noise exposed experimental groups showed that the previous increase in peak intensities for K and Ca observed in the noise-exposed subjects (Figure 8) had returned to intensities comparable to control levels upon administration of α-lipoic acid (Figure 11). Whereas there were no detectable differences in peak intensities for Fe and Zn upon administration of α-lipoic acid prior to noise exposure. The peaks for Cu, S, P and Mo were still seen in the spectra for both experimental groups. The intensity of these peaks showed no detectable differences between groups.

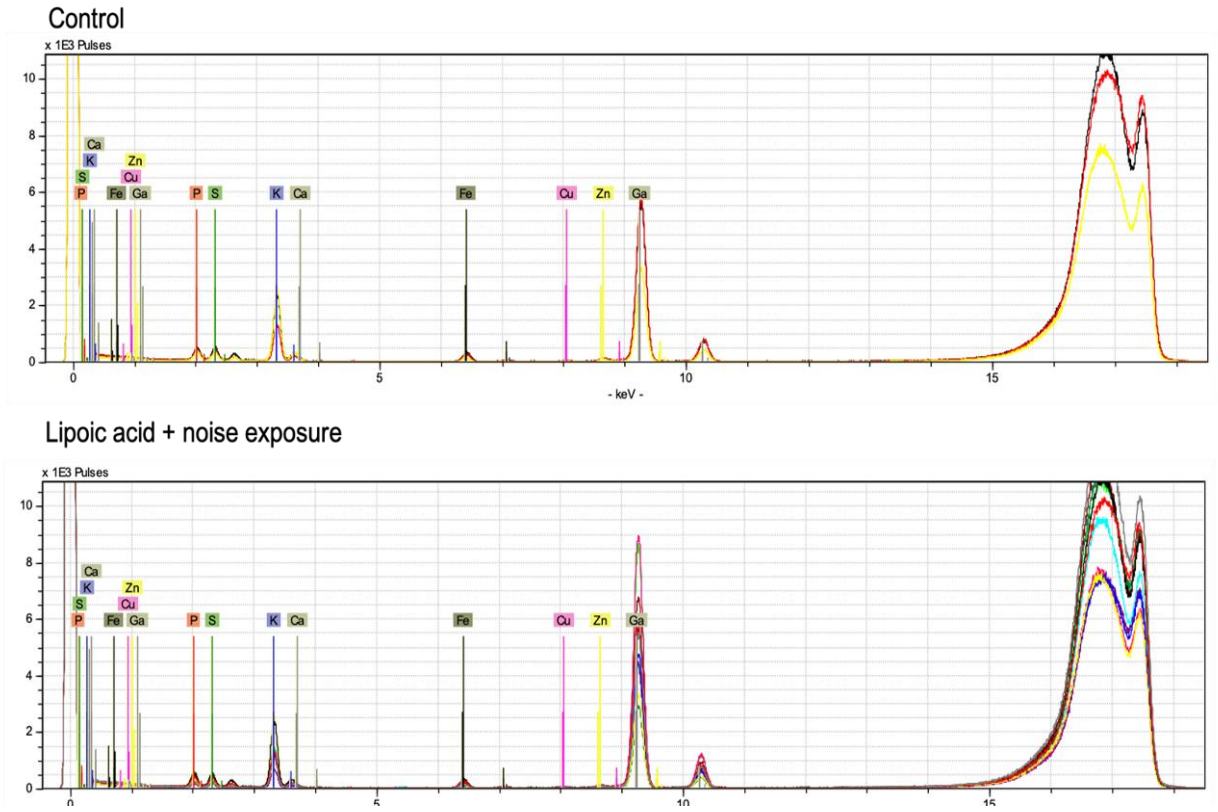


Figure 11: Representative spectra for control and α- lipoic acid groups. Each peak corresponds to an element detected in the sample. The intensity of the peak corresponds to the concentration of the specific element in the sample. From left to right, K peak was detected at about 3.25 keV, Ca detected at 3.5 keV, Fe detected at about 6.5 keV and Zn at about 9.0 keV, in all three representations. No shifts in peak positions were detected.

To provide further evidence that α- lipoic acid administration prior to noise exposure may have alleviated the noise-induced changes in biometal concentrations, the metal (Fe, Ca, K and Zn) concentrations in the control group (n = 5) were compared to those of the noise exposed group (n = 5) and the α-lipoic acid + noise exposed (n = 5). A Q-test was performed to exclude outliers. Non-parametric t-test analysis was performed between control and α- lipoic acid + noise exposed group. As seen in Figure 12 A-C, α- lipoic acid administration resulted in bringing back biometal concentrations to levels comparable to that of the control groups. Fe levels showed an increase from a mean of 5.0×10^{-5} μg/g in the control to 0.0003μg/g in the noise-exposed group (Figure 9A) and a reduction to 0.0001μg/g in the α- lipoic acid + noise-exposed group (Figure 12A). Ca exhibited a similar pattern from a mean of 0.0002μg/g in the control to 0.001μg/g in the noise-exposed (Figure 9B) and a reduction to 0.0003μg/g in the α- lipoic acid + noise-exposed group (Figure 12B). K increased from a mean of 0.0004μg/g in the control to 0.003μg/g in the noise-exposed group (Figure 9C) and a reduction to 0.0005μg/g in the α- lipoic acid + noise exposed group (Figure 12C). The changes observed in Zn concentration were non-significant from a mean of 3.8×10^{-5} μg/g in the controls to 5.3×10^{-5} μg/g in the noise-exposed group (Figure 9D) and remained relatively same at 5.9×10^{-5} μg/g in the α- lipoic acid + noise exposed group (Figure 12D).

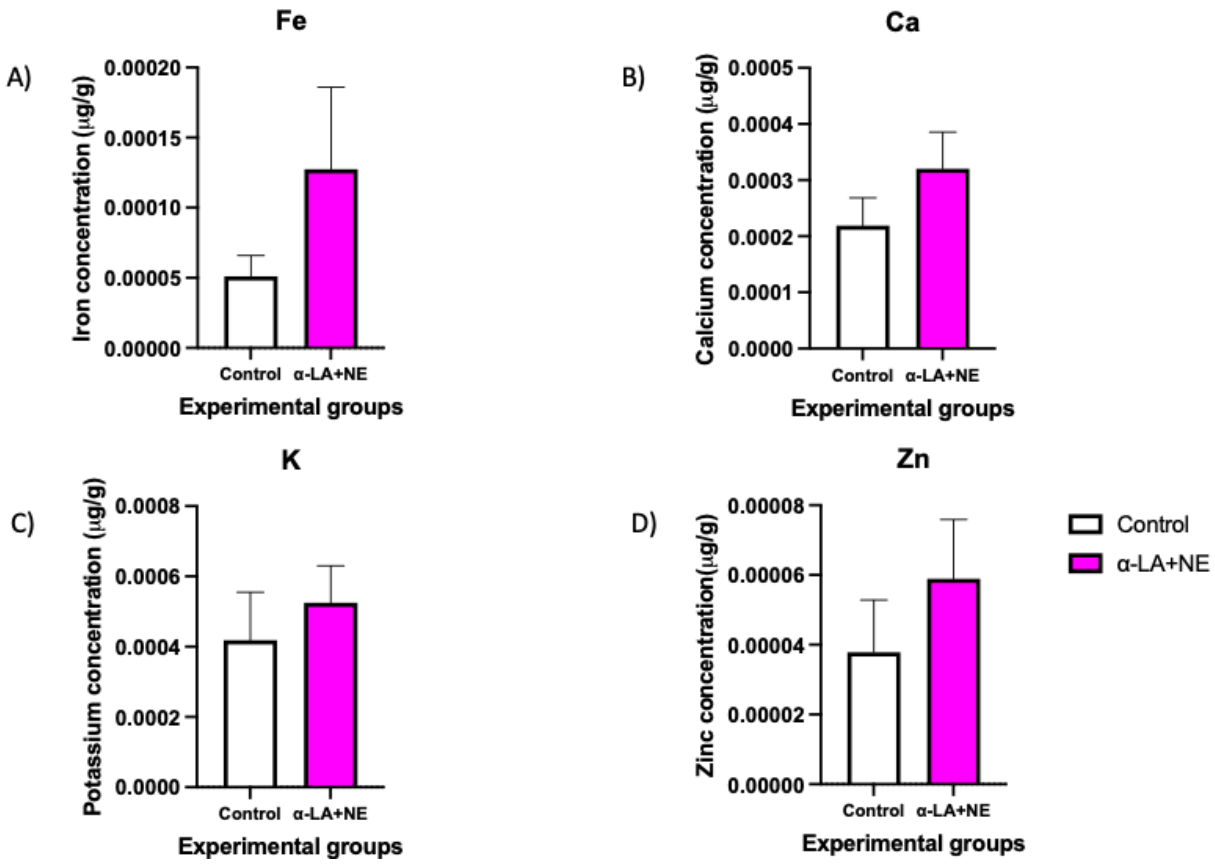


Figure 12: Effects of α-lipoic acid administration on concentration of biometals: Fe, Ca, K and Zn. A) Fe concentration comparable to control upon α-lipoic acid administration ($p = 0.1477$ B) Ca concentration comparable to control upon α-lipoic acid administration ($p = 0.1270$). C) K concentration comparable to control upon α-lipoic acid administration ($p = 0.2762$. D) Zn concentration comparable to control upon α-lipoic acid administration ($p = 0.1894$). α-LA+NE: α-lipoic acid + Same day noise exposure.

CHAPTER V

DISCUSSION

The present study was undertaken to evaluate changes in reactive oxygen species (ROS) as a plausible mechanism underlying the effect of noise on the dopamine system in the hub for central auditory processes, by using colorimetric assays and XRF analysis. Dopamine dysfunction has been implicated in neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. Patients diagnosed with these conditions also exhibit hearing deficit, thus attention has been drawn to the possible role of dopamine in auditory processes.

Loud Noise Triggers Production of Hydrogen Peroxide (H_2O_2) in the Inferior Colliculus

Recent data from our research laboratory, measured by fast scan cyclic voltammetry, has demonstrated that loud noise leads to decreased dopamine release in the inferior colliculus of adult Sprague-Dawley rats (Wilson, n.d., p. 50). It has previously been shown that overproduction of ROS is a major contributor to noise-induced hearing loss (Park et al., 2014). Reactive oxygen species can also modulate neuronal processes, including synaptic dopamine release via regulated export using the Calcium (Ca^{2+}) channels pre-synaptically (Chen et al., 2001) and the ATP-sensitive K^+ (K_{ATP}) channels post-synaptically (Rice, 2011). However, there is a research gap that still exists in understanding the mechanism underlying how noise impacts on the dopamine system.

In the present study, HRP colorimetric assay revealed that loud noise exposure evoked the formation of ROS, specifically H_2O_2, in the inferior colliculus of the adult rat brain. Exposure to deafening noise for four hours increased the H_2O_2 concentration in the inferior colliculus by

more than two-fold. Because hydrogen peroxide can act as a signaling molecule by modulating synaptic dopamine release (Chen et al., 2001), it is likely that the excessive production of H_2O_2 is responsible for the downregulation of synaptic dopamine release observed in the inferior colliculus following noise exposure. Based on this finding, we postulate that the noise induced elevation in H_2O_2 will activate ATP-sensitive K^+ (K_{ATP}) channels and shuts down calcium channels thus inhibiting dopamine neuron firing in the inferior colliculus (Chen et al., 2001; Rice, 2011). Elevated hydrogen peroxide levels can also alter the activation and the conformation of tyrosine hydroxylase and subsequently diminish dopamine synthesis and release (Daubner et al., 2011). Altogether, the data demonstrate that deafening noise leads to oxidative stress that could impact dopamine neurotransmission in the inferior colliculus.

No Changes in Adenosine Triphosphate (ATP) Levels Observed Following Exposure to Deafening Noise

We hypothesized that increased H_2O_2 produced as a result of excessive noise exposure may lead to the depletion of ATP as a result of the detoxication process. Furthermore, ATP is required to activate Protein Kinase A (Ford, 2014). Therefore, we suggested that ATP depletion disables the activation of Protein Kinase A and thus, decreases tyrosine hydroxylase activation.

To better understand this proposed mechanism by which noise exposure can result in the attenuation of dopamine release, ATP levels in noise exposed subjects were examined and compared to their controls. Unlike the significant increase in H_2O_2 observed, there were no significant changes recorded for ATP concentration between the control and noise-exposed subjects.

To shed more light on this observation, we must look at the energetics of adenosine triphosphate as they relate to living and dead organisms as well as the method sensitivity of the ATP colorimetric assay. Since ATP is the energy currency of the cell, its turnover in actively

respiring tissue such as the brain is very fast. Therefore, as long as the animal is alive and in the absence of any pathology, the electron transport chain and oxidative phosphorylation pathways will quickly work to replenish lost ATP (Owen & Sunram-Lea, 2011). The 24-hour recovery period could have caused us to miss the window within which ATP changes could be accurately determined. During this time, any ATP loss will quickly be replenished as long as the subject had access to adequate nutrition. In addition, our inability to observe any differences in ATP could be due to the fact that the colorimetric assay may not have been a very sensitive. To verify these assertions, future experiments should narrow the recovery time before sacrifice and ATP analysis. Single molecule tracking of ATP in live animals to determine the differences in ATP levels between control and noise exposed rats, may also be undertaken. Another a high-resolution method which may be used is Magnetic Resonance Spectroscopy (MRS). Recent evidence obtained by magnetic resonance spectroscopy has shown a link between NAD/NADH ratio and ATP levels in neurological diseases such as Schizophrenia and Bipolar disorder (Cuenoud et al., 2020). In light of this finding, our hypothesis should be further explored in the context of noise-induced hearing loss. However, the present findings point to no significant change in ATP levels following noise exposure.

Loud Noise Modulates the Levels of Biometals Essential for Biosynthesis and Neurotransmission

Our hypothesis also suggests a decrease in dopamine synthesis as a result of excessive production of ROS from the noise exposure. Emerging evidence has shown that biometals are involved in neuromodulation (Benedictis et al., 2019; Grochowski et al., 2019; Huidobro-Toro et al., 2008). To determine the effect of deafening noise on proteins that regulate dopamine synthesis, biometals that act as prosthetic groups and may be involved in protein coordination and neurotransmission were examined with XRF. Analysis was performed for Fe, Ca, K and Zn

ions. The K peak detected, at about 3.25 keV, showed a higher peak intensity in the noise-exposed group than control sample. Concurrently, the Ca peak detected at about 3.5 keV also showed a higher peak intensity. The peaks examined for Fe and Zn remained relatively same in noise exposed as compared to controls.

Quantitative analysis of the XRF data revealed a significant increase in Fe concentration following noise exposure. Iron accumulation has been reported in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Huntington's diseases (Zecca et al., 2004). Iron accumulation has also been observed in the natural aging process as iron-induced oxidative stress may result in neurodegeneration (Zecca et al., 2004). Iron is found bound by metalloproteins where they are tightly regulated. Therefore, it is plausible that the excessive production of H_2O_2 produces a redox environment where these metalloproteins are oxidized thus releasing Fe. Additionally, in the presence of Fe, H_2O_2 is converted into the hydroxyl radical which causes extensive protein, DNA and membrane damage (Phaniendra et al., 2015). By this, it can be concluded that noise exposure results in the formation of H_2O_2 which in the presence of increased iron concentration exacerbates the oxidative stress produced from noise exposure.

Potassium and calcium levels in the noise exposed group showed interesting trends in comparison with the control groups. The results of the analysis demonstrated there was a significant increase in these two biometals upon noise exposure. It has been previously shown that Ca^{2+} and K^+ control the voltage gated channels that allow the release of dopamine with the Ca^{2+} channels acting pre-synaptically and the K^+ channels acting post synaptically (Chen et al., 2001; Rice, 2011). A delicate balance is required between these two in order to achieve dopamine release and uptake. Therefore, any changes in concentration of Ca^{2+} and K^+ will likely alter the dopamine response. This observation could also be correlated with a previous study

which showed elevated K^+ concentration could be an indicator of brain tissue injury (Antunes et al., 2014). Another study also demonstrated that traumatic brain injury causes elevated Ca^{2+} in patients (Sun et al., 2008). Together, these research conclusions in light of our current findings point to the fact that not only could the altered concentrations of Ca^{2+} and K^+ attenuate dopamine release but may very well serve as an indicator of nerve cell injury or death. This provides a possible link between ROS produced as a result of noise exposure and neuronal cell death in the inferior colliculus which may ultimately lead to hearing loss.

Zinc did not show any significant changes between noise-exposed and control subjects upon mean comparison. Our observation is contrary to existing literature which shows an increase in Zn observed in neurodegeneration (Benedictis et al., 2019; Leskovjan et al., 2011). Zinc has been shown to be a neuromodulator and seemingly, excessive loud noise which produces ROS may be involved in depolarizing the synaptic vesicles which contain Zn in the inferior colliculus (Leskovjan et al., 2011). In the context of noise-induced hearing loss, our observation demonstrates that increased ROS may disturb the neurological biometal concentrations in a manner independent of Zn.

The findings in the present work suggest that the production of ROS as result of noise exposure may cause at the very least a transient imbalance in the concentrations of biometals resulting in the attenuation of the dopamine response. However short this transient period may be, it is possible that this could be one of the reasons dopamine release is severely impaired upon exposure to noise. The transitory nature of the changes in the biometal concentrations cannot be excluded and as such a 24-hour recovery period may not be long enough to conclude on the differences between the control and the noise-exposed subjects. As a recommendation, future work should focus on increasing the number of subjects and performing time-point experiments.

This will help ascertain clear-cut differences and characterize the dynamic nature of neurotransmission as relates to biometal concentrations.

Antioxidant, α-Lipoic Acid can Mitigate some of the Effects of Loud Noise in the Inferior Colliculus

To verify whether the administration of the antioxidant α- lipoic acid will prevent the noise-induced impact on the dopamine system, the levels of H_2O_2 and ATP were measured. In addition to this, biometal analyses of Fe, Zn, K and Ca in the brain tissues of the α- lipoic acid subjects were done using XRF spectrometry. The results obtained revealed that H_2O_2 concentration reduced to a level comparable to that of the controls upon the administration of α-lipoic acid immediately before noise exposure. In the context of noise-induced hearing loss, it is plausible that the noise-induced impact on the dopamine system could be alleviated by prior α-lipoic acid administration. This suggests that, as per our hypothesis, ROS plays a central role in the mechanism of noise-induced hearing loss. However, the experiment revealed that administering α- lipoic acid 24 hours prior to noise exposure had little to no effect on ROS. This implies that in order for α- lipoic acid to effectively scavenge for ROS, it should have been administered shortly before noise exposure. Administering α- lipoic acid 24 hours prior to noise exposure did not render its antioxidant effect useless rather the effect against ROS was severely reduced. However, analysis of adenosine triphosphate levels showed that α- lipoic acid further decreased the amount of ATP, although the changes were non-significant. This observation could be attributed to two things. First, the process of injecting subjects with α- lipoic acid is traumatic to the animal therefore energy may be expended to repair the damage caused at the site of injection and the inflammation that occurs as a result. Second, the thiol groups in α- lipoic acid may be interacting with ATP synthase thereby resulting in its oxidation and subsequent reduction

in activity. This observation is also supported by a recent publication where it was observed that thiol groups reversibly oxidize the F_1-Fo subunit of ATP synthase thus causing its inactivation in *X. laevis* oocytes (Cobley et al., 2020). Therefore, in order to draw an accurate picture of the relationship between H_2O_2 and ATP, another method of ATP analysis such as MRS or another antioxidant which does not interfere with the action of ATP synthase may be used.

Qualitative analysis of the XRF spectra for Fe, Ca, K and Zn was performed. No change in peak intensities was observed for the α- lipoic acid + noise exposed representative spectra for Fe and Zn. The reason for this observation may be due to these ions possessing a relatively greater number of shielding electrons which may make any slight changes in peak intensity challenging to analyze qualitatively. Administration of α- lipoic acid reduced the peak intensities observed for K and Ca in the noise exposed group, returning the peak intensities to levels comparable to the control. This observation suggests that upon noise exposure the increase in K may be as a result of damage to the post-synaptic terminals which control dopamine release, a consequence of neuronal cell death (Antunes et al., 2014). In agreement with our hypothesis, this finding provides a clue that noise exposure causes dopamine dysfunction demonstrated by the observation that prior administration of α- lipoic acid prevented any noise-induced damage thus keeping K and Ca concentrations at basal levels. The peak intensity for Zn remained relatively same upon administration of α- lipoic acid. Many studies have shown that Zn supplementation improves tinnitus in patients experiencing noise-induced hearing loss (Hwang, 2016; Person et al., 2016; Yeh et al., 2019). While literature is silent on the reason for Zn supplementation in these subjects, our present findings do not support Zn therapy for patients with tinnitus.

Furthermore, quantitative XRF analysis of the biometals: Fe, Ca, K and Zn showed that α- lipoic acid administration brought the concentrations of Fe, Ca and K to levels comparable to

that of the control group. On the other hand, Zn remained at a concentration relatively comparable to that of the noise exposed group following the administration of α- lipoic acid. The findings in this experiment demonstrated the relationship between biometals and ROS. The administration of α-lipoic acid alleviated the effect of noise exposure on biometal concentration. The fact that no significant changes were observed in Zn concentration further emphasizes the point that increased ROS may disturb the neurological biometal concentrations in a manner independent of Zn. Due to the fact that α- lipoic acid administration maintained the concentrations of Fe, Ca and K at levels comparable to the control, it is safe to implicate excessive ROS produced as a result of noise exposure as being responsible for attenuated dopamine release. In this context, we can assume α- lipoic acid administration prior to noise exposure causes a dynamic change to the redox environment in the inferior colliculus by scavenging for ROS. To determine which of these biometals are the most affected due to the pathological effect of ROS on the dopamine system, future experiments should evaluate the concentrations of these biometals by undertaking redox-affinity blotting. This will shed light on all ions especially those bound by metalloproteins and will give a clearer picture of the effects of reactive oxygen species on essential biometals required for neurotransmission.

Conclusion

This study was undertaken with the aim of evaluating the effect of noise on the production of reactive oxygen species that could be an underlying mechanism of noise induce alterations in dopamine neurotransmission in the inferior colliculus.

The study supports the hypothesis that excessive noise exposure produces ROS in the inferior colliculus of the adult rat brain. Herein, the evidence that supports the findings have been presented. First, we have shown significant increase in H_2O_2 in rats exposed to deafening noise

compared to their controls. In addition to previous research that shows that H_2O_2 is a dopamine neuromodulator, our present findings draw a strong correlation between elevation in H_2O_2 levels and the severe reduction of dopamine release in noise exposed subjects.

Although the present findings point to no significant change in ATP levels following noise exposure, choosing a more sensitive method of ATP analysis and/or narrowing the recovery time may show real time differences between control and noise-exposed subjects. Additionally, ROS produced as a result of noise exposure has the potential to attack dopamine neurotransmission at several points. Therefore, it is worth exploring alternative hypotheses such as the oxidative modification of TyrH or the gate-keeping G-proteins as plausible mechanisms bridging the gap between the observed ROS increase and the diminished dopamine response in noise-exposed subjects.

Second, the present data reveals that excessive production of H_2O_2 affects the concentrations of Fe, Ca and K. Thus, it can be inferred that H_2O_2 changes the redox environment thus changing biometal concentration of Fe. In addition to the oxidative damage that ROS elicits, the increase in Fe concentrations further compounds the oxidative stress that the auditory system of these rats are subjected to. In the presence of increased Fe, the conversion of H_2O_2 to the hydroxyl radical the most damaging form of ROS occurs at an even faster rate. The increase in Ca and K observed suggests that the oxidative stress on the central auditory system may result in neuronal cell death and alter dopamine neurotransmission. No significant changes were observed for Zn in our present work.

Third, we have shown that administration of α- lipoic acid prior to noise exposure has the potential of scavenging for ROS produced as a result of noise exposure. The next step will be to ascertain whether α- lipoic acid administration will prevent the decrease of dopamine release in

noise-exposed subjects. In our present work, prior administration of α- lipoic acid has no effect on restoring ATP levels. In addition, we recommend a more sensitive method of ATP detection which will accurately probe how excessive production of hydrogen peroxide modulates adenosine triphosphate levels in the inferior colliculus. Furthermore, it will be worth exploring other mechanisms through which ROS may affect dopamine neurotransmission, such as the oxidative modification of tyrosine hydroxylase. This will shed light on the underlying mechanism of noise-induced dopamine attenuation.

Furthermore, administration of α- lipoic acid prior to noise exposure has the potential of keeping biometal concentrations at levels comparable to the control thus providing a mechanistic clue to the relationship between reactive oxygen species and the neuromodulation of dopamine. Overall, this work has expanded our understanding of the dysregulation that occurs in the inferior colliculus as a result of exposure to loud noise. Based on the evidence obtained in this work in conjunction with previous work (Chen et al., 2001; Ford, 2014; Wilson, n.d.), we propose the following mechanism as shown in Figure 13 below.

Proposed mechanism

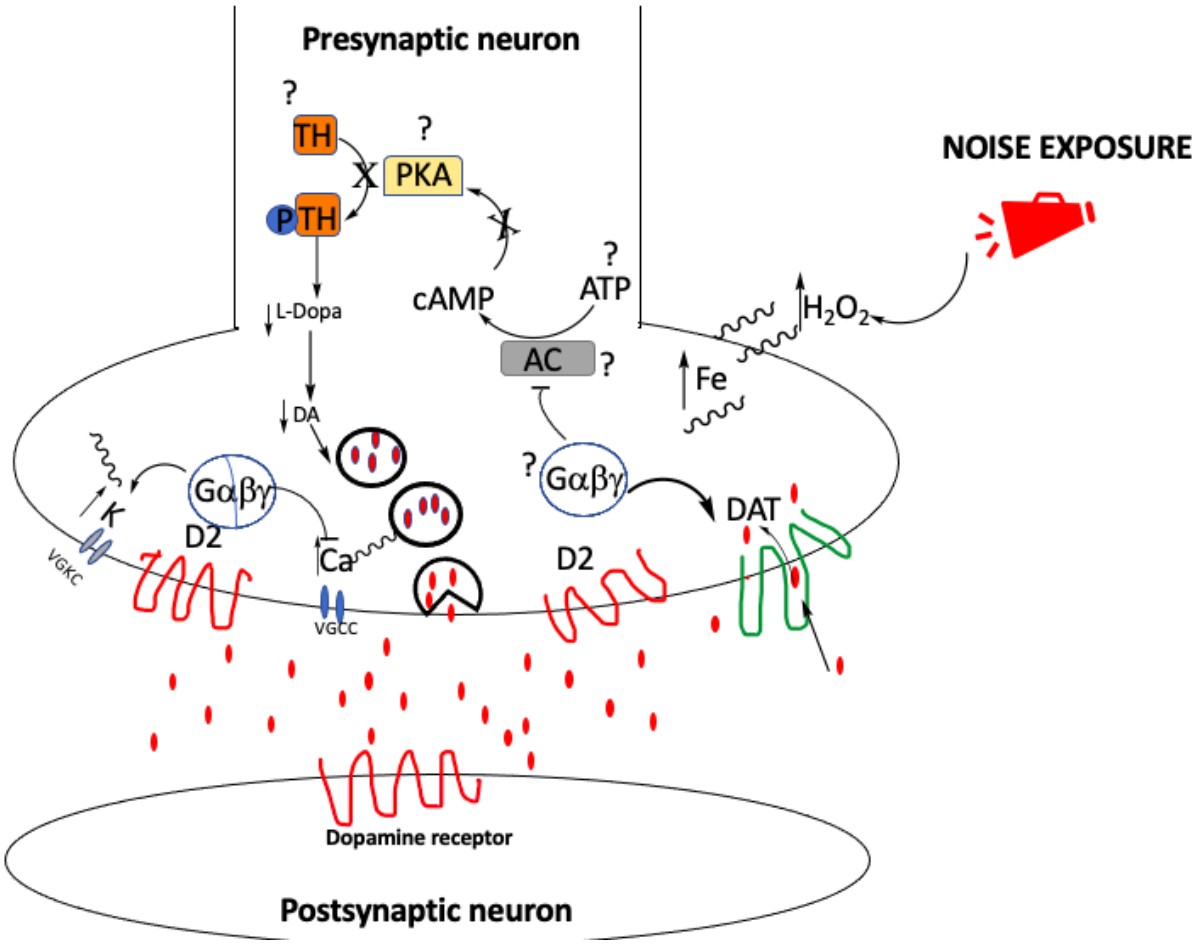


Figure 13: Proposed mechanism for inhibition of dopamine upon noise exposure. Loud noise produces H_2O_2 (represented by squiggly lines) in the neurons found in the inferior colliculus. H_2O_2 increases the concentration of Fe, Ca and K. Calcium channels are inhibited and potassium channels are activated, which may lead to diminished dopamine release. Other parts of the mechanism which may also be affected by excessive H_2O_2 produced as a result of noise exposure, are oxidative modification of Tyrosine Hydroxylase and Protein Kinase A, Adenylate Cyclase, G-proteins and ATP depletion (question marks represent portions of the mechanism for which the effects of H_2O_2 are unknown or unproven).

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