EFFECTS OF A CUPRIZONE DIET ON THE AUDITORY CORTEX OF C57BL/6 MICE

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By

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APPROVED

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Abstract

Multiple Sclerosis (MS) is the leading chronic degenerative disease in society today; the disease affects approximately 400,000 people in the U.S. and 2.5 million people worldwide. Furthermore, over 100,000 people between the ages of 15-50 are diagnosed with MS on an annual basis. It is widely known that MS targets the Central Nervous System (National MS Society). A defining feature in MS is demyelination concurrent with attack by the immune system (Coggan, 2015). It has long been known that axons are long, intermediate neurofilaments that send signals rapidly throughout the body. Fast signal conduction depends on the presence of myelin – lipid membrane sheets – that is tightly wrapped around the axonal body (Coggan, 2015).
The auditory cortex is composed of six regions and these regions communicate with each other via large, myelinated fibers to yield a hierarchical system that allows for complex auditory perception (Tsukano, 2016). Since MS targets myelin and the different regions of the auditory cortex communicate via myelinated fibers, MS will most likely alter auditory perception, which can be quantitatively measured via auditory brainstem responses (ABRs). ABR is an electroencephalographic response that maps out the central pathway of hearing, which is reflected through its waveform components along with providing statistical data about hearing. ABR consists of seven waveforms that correspond to different regions of the brain, however only the first three waveforms are reliable to accurately reveal sound processing ability in mice. Qualitative analysis to see the number and morphology of various types of neuromodulatory cells in the auditory cortex along with quantitative analysis to see the statistical data about hearing ability allows for determination of how hearing is affected as various durations of cuprizone administration (4 week cuprizone diet and 12 week cuprizone diet) are administered to determine the comprehensive effects of MS on the auditory cortex and hearing abilities.

Acknowledgments

I extend a special thank you to Dr. Khaleel Razak and his laboratory members for generously allowing us to share their workspace, and especially Ms. Anna Nguyen for her guidance during the acquisition and analysis of ABRs.

I also extend a thank you to the Tiwari-Woodruff lab for lending time and resources, and a special thank you to Hana Yamate-Morgan for being a mentor that I could always rely on.

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Introduction:

Multiple Sclerosis (MS) is the leading chronic degenerative disease in society today; the disease affects approximately 400,000 people in the U.S. and 2.5 million people worldwide. Furthermore, over 100,000 people between the ages of 15-50 are diagnosed with MS on an annual basis.

It is widely known that MS targets the Central Nervous System (National MS Society). A defining feature in MS is demyelination concurrent with attack by the immune system (Coggan, 2015). It has long been known that axons are long, intermediate neurofilaments that send signals rapidly throughout the body. Fast signal conduction depends on the presence of myelin – lipid membrane sheets – that is tightly wrapped around the axonal body (Coggan, 2015).

In addition to degradation of white matter, MS also yields degeneration and loss of a wide variety of cells such as parvalbumin neurons, astrocytes, microglia, oligodendrocyte precursor cells (OPCs), and mature oligodendrocytesIt is commonly known that neurons are the basic unit of the CNS. Parvalbumin protein in neurons serves as a Ca2+ buffer, exerting inhibitory effects by accerating the decay of Ca2+ transients, which can mediate neurotransmitter release (Caillard, 2000). Astrocytes have a wide variety of functions such as mediating synaptic function, aiding in development of white matter, and maintaining homeostasis to provide an appropriate environment for neuronal development (Sofroniew, 2010). As its name suggests, oligodendrocyte progenitor cells mature into oligodendrocytes and mature oligodendrocytes mediate the wrapping of myelin sheath around most axons (Purves, 2001). Microglia are the immune cells of the brain, which aid in developing the final neural circuitry during neurogenesis and have phagocytotic functions in the mature CNS (Wake, 2011). The fates of these cells are progressively visualized in the auditory cortex via fluorescent markers when performing IHC.

The auditory cortex is composed of six regions: anterior auditory field (AAF), primary auditory cortex (AI), the secondary auditory field (AII), dorsoanterior field (DA), dorsomedial field (DM), and dorsoposterior field (DP) – these regions communicate with each other via large, myelinated fibers to yield a hierarchical system that allows for complex auditory perception (Tsukano, 2016). Since MS targets myelin and the different regions of the auditory cortex communicate via myelinated fibers, MS will most likely alter auditory perception, which can be quantitatively measured via auditory brainstem responses (ABRs).

The auditory brainstem response is an electroencephalographic response that maps out the central pathway of hearing, which is reflected through its waveform components along with providing statistical data about hearing abilities such as threshold, interpeak interval, and amplitude. ABR consists of seven waveforms, however only the first three waveforms are reliable to accurately reveal sound processing ability in mice. Wave 1 corresponds to activity from the distal cochlear nerve, wave 2 corresponds to activity from the proximal cochlear nerve, and wave 3 corresponds to activity from the cochlear nucleus. Furthermore, abnormalities in the waveforms indicate abnormalities within the brain – delay or absence of wave 1 suggests cochlear lesion and prolongation of waves 1-3 interpeak latency suggests lower brainstem lesion (Tsukano, 2016).

Qualitative analysis to see the number and morphology of various types of neuromodulatory cells in the auditory cortex along with quantitative analysis to see the statistical data about hearing ability allows for determination of how hearing is affected as various durations of cuprizone administration (4 week cuprizone diet and 12 week cuprizone diet) are administered to determine the comprehensive effects of MS on the auditory cortex and hearing abilities.

Methods:

Animals/Tissue Groups:

Three groups of C57BL/6 mice were prepared. All mice were male and no more than 3 months apart in age. Group 1 was the control group and was fed a diet of normal chow and is referred to as "Nmls." Group 2 was fed a cuprizone diet for 4 weeks and is referred to as "4wk Cpz" and Group 3 was fed a cuprizone diet for 12 weeks and is referred to as "12wk Cpz." Refer to Table 1 for more details.

Strain	Cage ID	SEX	MARK	Code	DOB	Disease Start	Groups (short)	Notes	Euth Date	Fate
Rapamycin Project Filler Mice										
C57BL/6	CM67		NE		1/14/2016	N/A	Nmls		8/10/2016	ABR/IHC
			RP		1/4/2016	N/A			8/10/2016	ABR/IHC
C57BL/6	CM68		NE		2/11/2016	7/6/2016	4wk Cpz		8/10/2016	ABR/IHC
			RP		2/11/2016	7/11/2016			8/10/2016	ABR/IHC
C57BL/6	CM72		NE		3/4/2016	5/12/2016	12wk Cpz		8/10/2016	ABR/IHC
			RP		3/4/2016				8/4/2016	ABR/IHC
C57BL/6	CM94		NE		4/1/2016	N/A	Nmls		2/10/2016	ABR/IHC
										ABR/IHC
										ABR/IHC
										ABR/IHC
										ABR/IHC
C57BL/6	CM95	M	NE		4/1/2016		Nmls		8/10/2016	ABR/IHC
										ABR/IHC
										ABR/IHC
										ABR/IHC
										ABR/IHC

Table 1. Experimental data sheet for the mice used and treatment administered.

ABR Recordings:

ABR recordings were performed inside a sound-attenuating chamber in collaboration with the Razak laboratory at UCR. Mice were anesthesized with isoflurane and placed on a Kopf model small animal stereotaxic instrument. The positive (active) electrode was placed at the vertex, the negative (reference) electrode was placed in the right cheek, and a ground electrode was placed in the left cheek. Then, click sounds were presented from a speaker 10cm away from the mouse ear to determine hearing threshold. Mice were presented with 100 μs click stimuli between 80 and 10 dB SPL, decreasing in 10 dB SPL (sound pressure level) steps.

Tissue Processing

Following the recordings, mice were provided an overdose of isoflurane inhalation and then perfused with 1X PBS, followed by 10% formalin. Brains were collected, postfixed overnight, and then cryoprotected. Next, brains were embedded in gelatin blocks to minimize damage during sectioning. First, a layer of liquid gelatin was added to the bottom of an embedding mold and allowing to solidify. Then, the brain was placed on the gelatin layer and the embedding mold with brain and securing drops of gelatin were then refrigerated. Finally, a last layer of gelatin was placed on top of the brain and the embedding mold was placed back in the 4-degree Celsius refrigerator. Once the gelatin solidified, the block containing brain was gently cut out of the embedding mold, and the gelatin block was fixed in 10% formalin for24 hours, and then cryoprotected with 30% sucrose for 48 hours. Finally, gelatin-embedded brains were sectioned coronally at a thickness of 40μm using a cryostat. Four tissue sections corresponding to Bregma coordinates -2.8 (anterior) and 4 tissue sections corresponding to Bregma coordinates -3.2 (posterior) in the Mouse Atlas were selected from the control treatment group, 4wk Cpz treatment group, and 12wk Cpz treatment group.

Immunohistochemistry:

IHC was performed to identify different cell types (listed in Table 1) with fluorescent markers in order to visualize changes within 4- and 12- week cuprizone groups compared to the normal group. Table 2 provides a comprehensive list of antibodies used in IHC. Images were then taken by confocal microscopy where 6 40X images were taken of the right hemisphere of the brain (auditory cortex). All images were then imported to ImageJ for analysis where number and morphology of each cell type was thoroughly analyzed.

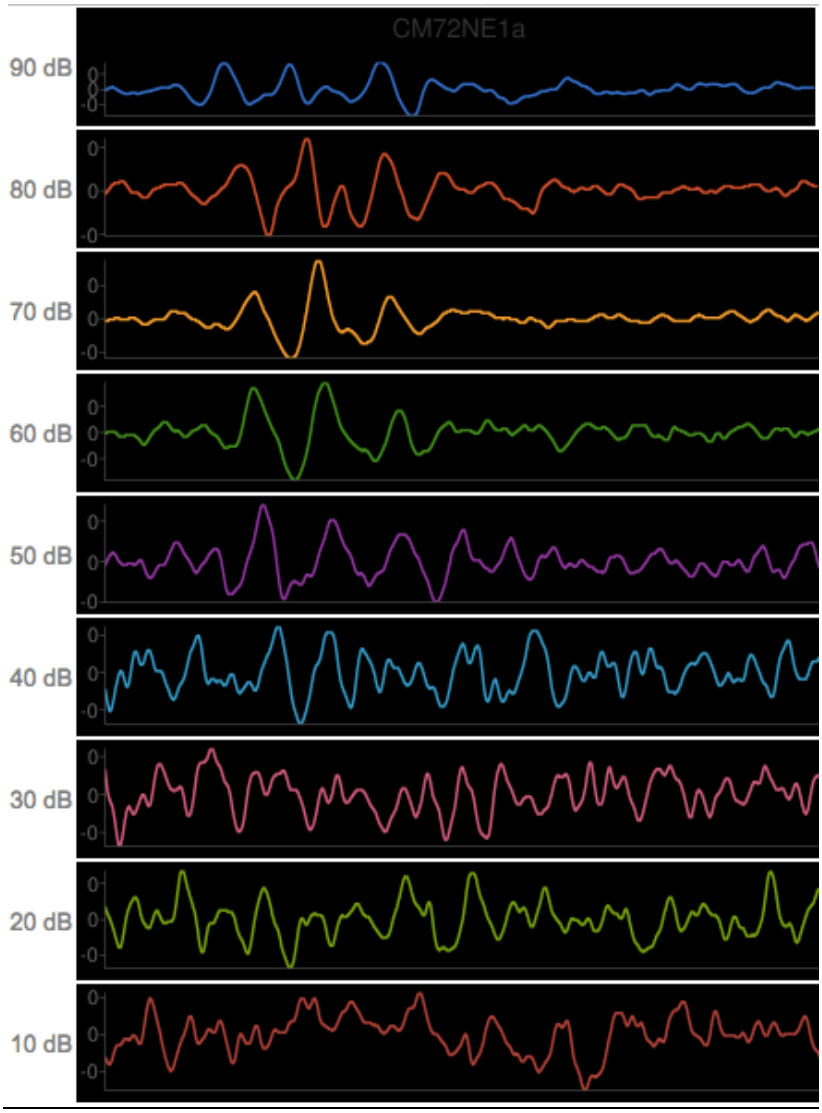
Antibody Name	**Target**	**Vendor**	**Catalog**
Neun	Neuron	Millipore	ABN78
PV	Parvalbumin	Millipore	MAB1572
DAPI	Nucleus	Millipore	268298
CC1	Mature myelinating oligodendrocytes	GeneTex	GTX16794
Olig2	Oligodendrocytes (OPCs)	Millipore	AB9610
GFAP	Glial fibrillary acidic protein; Astrocytes	Zymed	18-0063
Iba1	Microglia	Millipore	MABN92

Table 2. Details about the antibodies used and their targets

ABR Analysis:

Frequency (x-axis) and dB (10 dB – 90 dB in 10 dB intervals) (y-axis) were plotted to yield auditory brainstem response graphs from which threshold, peak latency, and peak amplitudes were determined. Threshold was identified by determining the lowest dB level that yielded visible waveforms 1-3 from the ABR graphs produced.

A



B

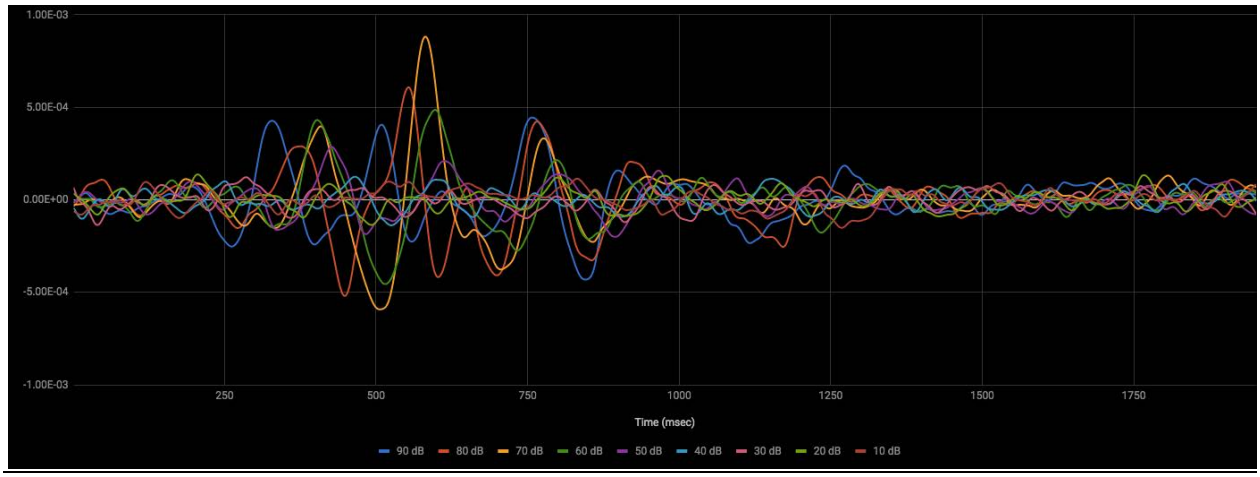


Figure 1. Computative auditory brainstem response recordings from the auditory cortex.

The brainstem responses to a pure tone ranging from 10dB – 90dB were recorded and graphed. The graphs reveal threshold in terms of what decibel level peaks 1-3 are clearly visible on the graphs. Furthermore, peak latency, interpeak interval, and amplitude can also be calculated.

Analysis:

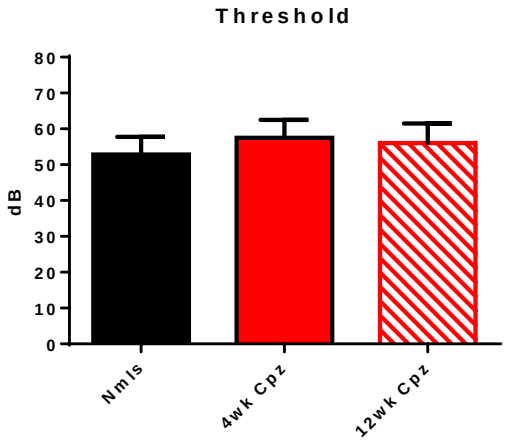


Figure 2. Threshold levels for three different groups of mice. The normal group was fed a chow diet, the 4wk Cpz group were fed a cuprizone diet for four weeks, and the 12wk Cpz group were fed a cuprizone diet for 12 weeks. All three groups of mice were exposed to a click stimulus at increasing sound pressure level (SPL) and their response was recorded via electrode recordings. The lowest SPL that elicited an electrical response was recorded to be threshold. On the ABR graphs produced, threshold levels were identified by determining the lowest 10dB interval, which clearly produced waveforms 1-3.

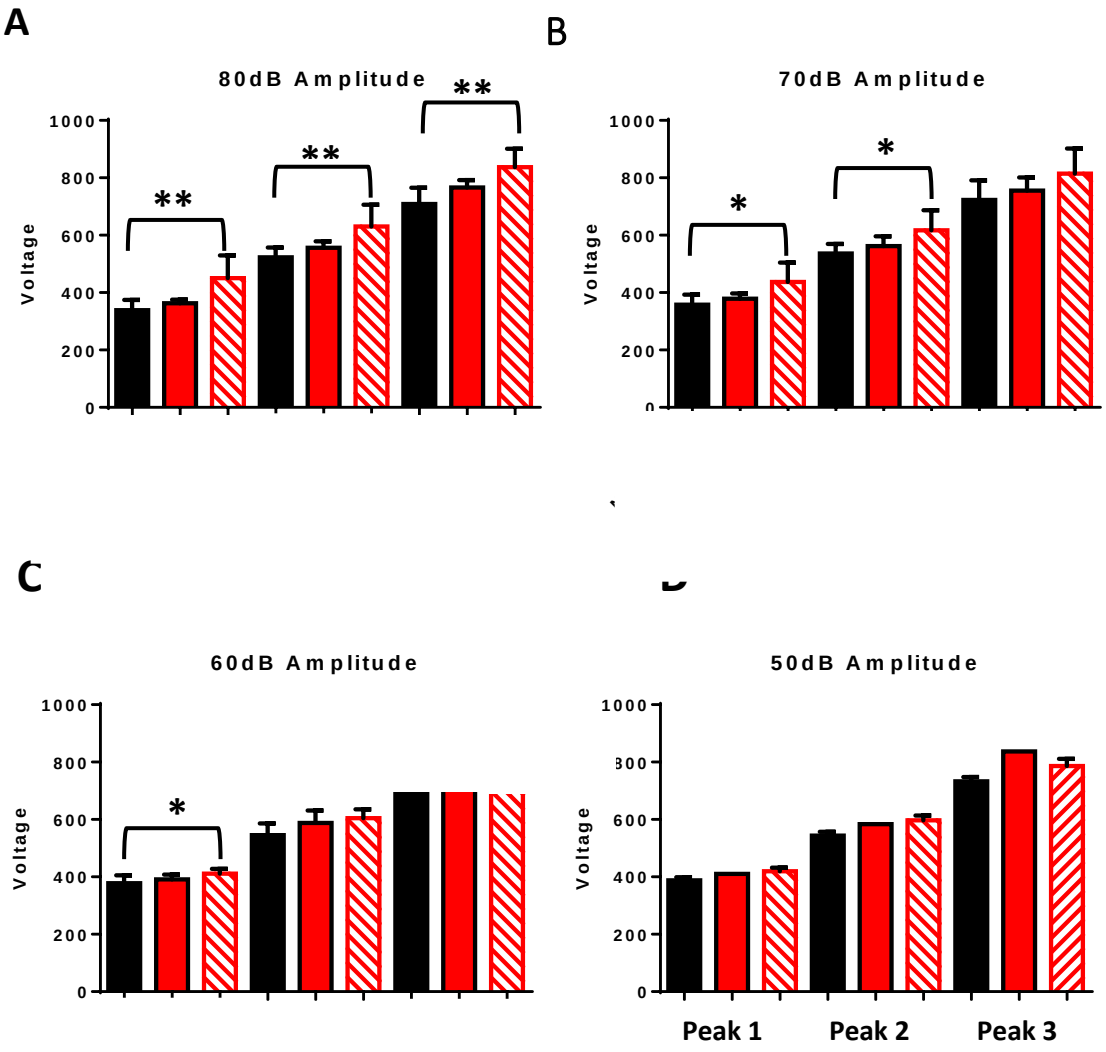


Figure 3. Amplitude of waveforms 1-3 in Nrml, 4wk Cpz, and 12wk Cpz mice at every 10dB interval beyond threshold. The amplitude of each peak was measured via a grid system in which the value at the point corresponding to the highest and most centered point of the peak was labeled as amplitude. Only peaks 1-3 were measured because they were the most consistent in producing stereotypical waveform.

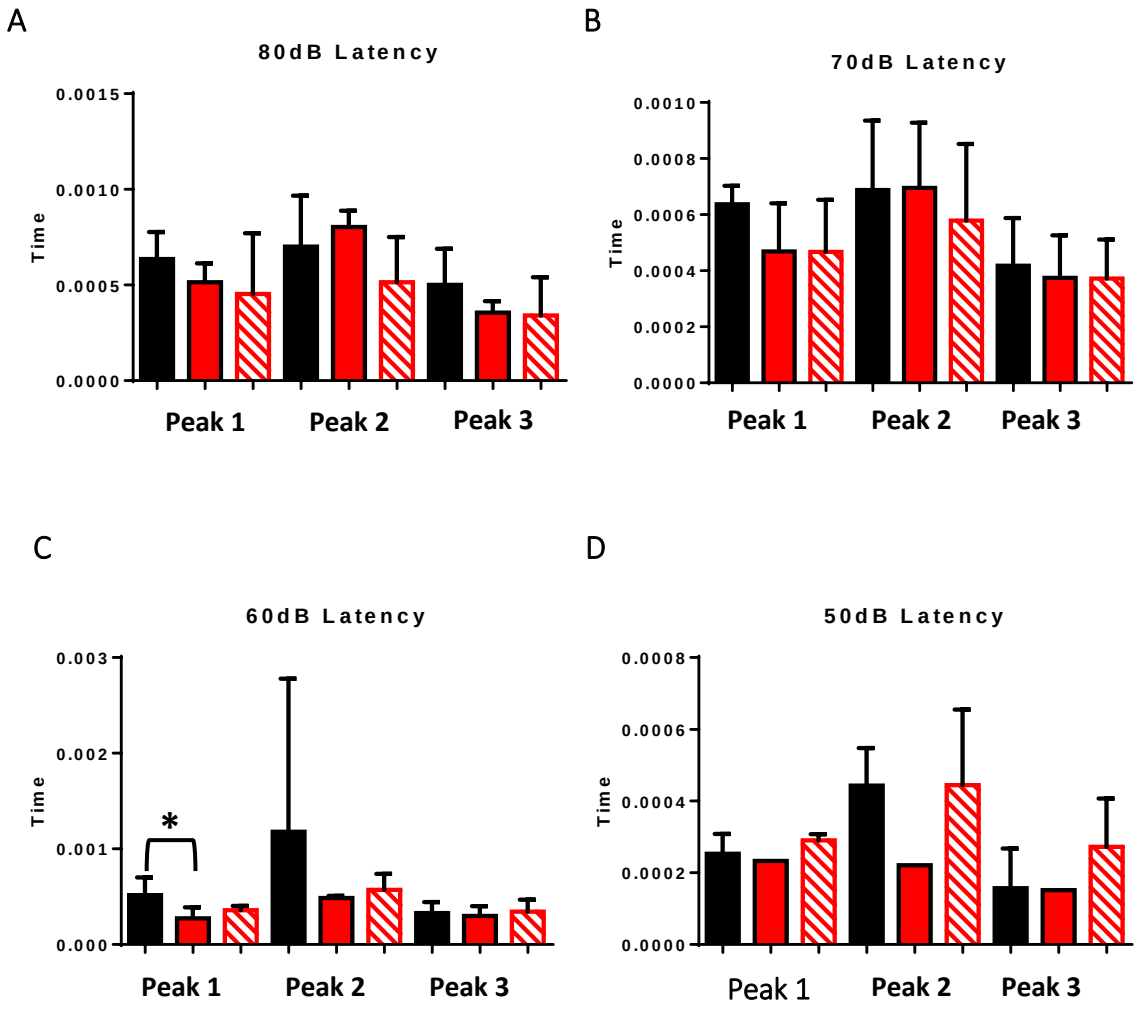


Figure 4. Latency of peaks for Nrml, 4wk Cpz, and 12wk Cpz mice. The latency, or time, at the onset of each peak was determined by measuring the time at which amplitude of each peak was recorded. Only latencies for peaks 1-3 were regarded since peaks 1-3 had the most stereotypical waveforms.

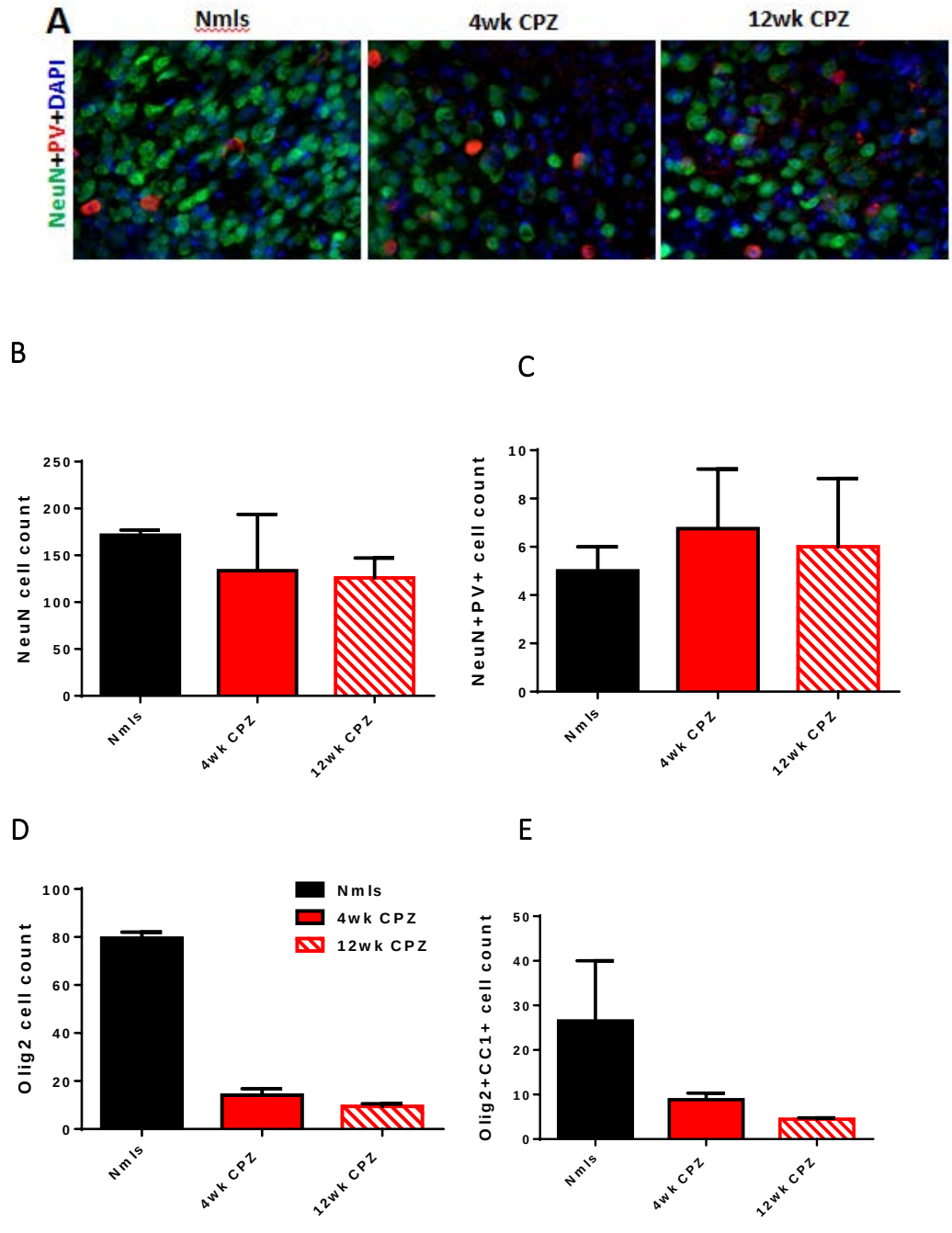


Figure 4. Numbers of oligodendrocytes decrease in the auditory cortex with increasing duration of cuprizone administration. Six images at 40X magnification were taken of the auditory cortex from 4 sections of each treatment group. A. The representative images from each group are shown (green: Neun, red: parvalbumin, blue: DAPI). B-C. The number of neurons in

each treatment group were counted using ImageJ. D-E. The number of oligodendrocytes were counted.

Results/Discussion:

Threshold level increased in 4wk cuprizone group and 12wk cuprizone group compared to the normal group, suggesting that cuprizone decreases the lowest SPL that can be heard. Cuprizone administered for 4 weeks and 12 weeks resulted in the hearing threshold to be 60dB, while threshold for Nmls (no cuprizone diet administered) was approximately 50dB.

As expected in accordance to stereotypical ABR waveforms, amplitude increased with each progressive waveform. Interestingly, though, amplitude was highest for 12wk cuprizone group and lowest for the normal group, A statistical significance was observed between the normal group and 12wk cuprizone group in 80dB for all three peaks, 70dB for the first two peaks, and 60dB for the first peak.

Latency was consistent in all of the peaks and no clear pattern was revealed. However, there was a statistical significance between the normal group and 12wk cuprizone group at 60dB.

IHC and cell counting revealed that oligodendrocytes decreased as duration of the cuprizone treatment increased. Normal group had the greatest number of oligodendrocytes, while 4wk cuprizone group had a lower number of oligodendrocytes and 12wk cuprizone group had the least number of oligodendrocytes. Preliminary analysis also showered that OPC cells counts, parvalbumin cell counts, glial cell counts, and astrocytes cell counts were also lower suggesting that cuprizone causes a decrease in cell types associated with creating and maintaining myelin.

Conclusion:

The quantitative and morphological results were what we expected them to be with the exception of amplitude. We expected amplitude to decrease as duration of the cuprizone treatment increased since hearing should decrease with increased degradation of white matter, however the opposite occurred. This experiment was very preliminary – we have not been able to find much research about the auditory cortex of mice or how damage of white matter affects the auditory cortex or hearing. Quantitative analysis via auditory brainstem response graphs did show that cuprizone treatment causes a decrease in hearing abilities of mice. Furthermore, increased duration of cuprizone treatment causes increased hearing loss. A similar conclusion was derived from morphological analysis via IHC and cell counting – cuprizone treatment causes a decrease in various different cell types such as mature oligodendrocytes, oligodendrocyte progenitor cells, astrocytes, glial cells, and parvalbumin cells and cell counts decrease with increasing duration of the cuprizone treatment.